Tubulysin derivatives and methods for preparing the same

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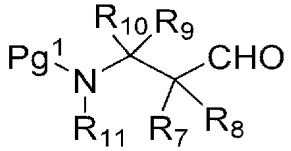
A



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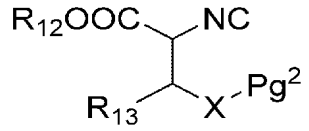

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(57) Abstract: The invention relates to novel means and methods for the synthesis of tubulysin and derivatives thereof, which find their use e.g. as cytotoxic agents in targeted drug delivery. Provided is a method for preparing a tubulysin derivative, comprising reacting compounds A, B and C in a 3-component Passerini reaction, wherein compound A is a carboxylic acid according to the general formula (A); wherein compound B is an aldehyde according to the general formula (B); and wherein compound C is an isocyanide according to the general formula (C).

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Title: Tubulysin derivatives and methods for preparing the same.

The invention relates to the field of medicinal chemistry. In particular, it relates to novel means and methods for the synthesis of tubulysin and derivatives thereof, for use as cytotoxic agents e.g. in targeted drug delivery.

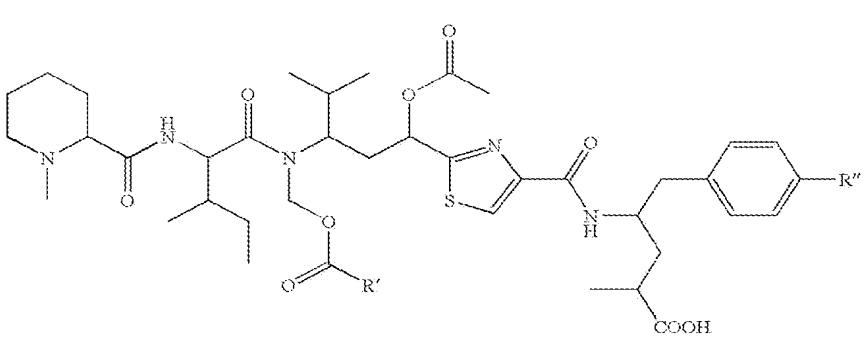
Antibody-drug conjugates or ADCs are an important class of highly potent biopharmaceutical drugs designed as a targeted therapy for the treatment of cancer. Unlike chemotherapy, ADCs are intended to target and kill only the cancer cells and spare healthy cells. ADCs are complex molecules composed of an antibody linked to a biologically active cytotoxic (anticancer) payload or drug. Antibody-drug conjugates are examples of bioconjugates and immunoconjugates. By combining the unique targeting capabilities of monoclonal antibodies with the cancer-killing ability of cytotoxic drugs, antibody-drug conjugates are designed to allow sensitive discrimination between healthy and diseased tissue. This means that, in contrast to traditional chemotherapeutic agents, antibody-drug conjugates should selectively target and attack the cancer cell so that healthy cells are less severely affected.

A stable link between the antibody and cytotoxic agent is a crucial aspect of an ADC. A highly stable ADC linker will ensure that less of the cytotoxic payload falls off in circulation, driving an improved safety profile, and will also ensure that more of the payload arrives at the cancer cell, driving enhanced efficacy. Linkers are based on chemical motifs including disulfides, hydrazones or peptides (cleavable), or thioethers (noncleavable) and control the distribution and delivery of the cytotoxic agent to the target cell.

Highly toxic small molecules or natural products have recently found important commercial applications in ADCs. Currently, dolastatine, maytansin and other natural product derivatives are coupled to antibodies to increase their efficacy. Several of such compounds are marketed already and multiple are in clinical trials. (Maturing antibody–drug conjugate pipeline hits 30 Asher Mullard, Nature Reviews Drug Discovery 12, 329–332 (2013) doi:10.1038/nrd4009).

However, current ADC suffer from a number of drawbacks. For example, there is only a limited number of useful drugs, and some induce toxic side effects based on the toxins, the inappropriate linkers, and/or the inappropriate targets/antibodies.

The tubulysins, first isolated by the Hofle/Reichenbach group from myxobacterial cultures are exceptionally potent cell-growth inhibitors that act by inhibiting tubulin polymerisation and thereby induce apoptosis. See Sasse et al. J. Antibiot. 2000, 53, 879-885; WO98/13375. These compounds show high cytotoxicity in the low picomolar IC_{50} in a panel of cancer cell lines and are thus of interest as potential anticancer therapeutics. Tubulysins (I) are tetrapeptides, containing three unusual amino acids; thus, the total synthesis poses a considerable challenge to organic chemists.



(I)

- Tubulysin A: R′=$CH_2CH(CH_3)_2$; R″=OH
- Tubulysin B: R′=$CH_2CH_2CH_3$; R″=OH
- Tubulysin C: R′=CH_2CH_3; R″=OH

- Tubulysin D: R′=$CH_2CH(CH_3)_2$; R″=H
- Tubulysin E: R′=$CH_2CH_2CH_3$; R″=H
- Tubulysin F: R′=CH_2CH_3; R″=H

The extremely high cytotoxicity of some tubulysins also has the disadvantage that a high general toxicity as well as a low selectivity against normal cells is observed. In an attempt to lower the toxicity of the tubulysins and to enhance their selectivity, polymer conjugates and bioconjugates of tubulysins were developed (see e.g. WO2004/005326) which exhibit a higher selectivity by a given cytotoxicity as well as lower toxicity as compared to the unconjugated compounds. Herewith, predominantly cancer cells are targeted in the human and the animal body and healthy tissue is not affected.

Thus, the therapeutic potential of tubulysin derivatives and (antibody) conjugates is very promising. Unfortunately, tubulysin derivatives are very difficult to obtain by fermentation because of poor yields and cumbersome isolation procedures. Whereas different approaches are available for their complete chemical synthesis, these are quite lengthy and provide a low overall yield.

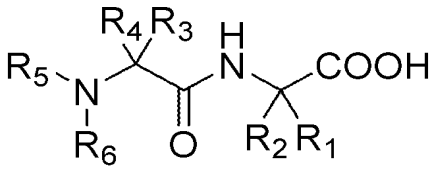
The present inventors therefore set out to develop a new synthetic approach towards potent novel tubulysin derivatives. In particular, they aimed for a synthetic procedure which is short (in numbers of steps), cheap, allows for full stereoisomer control and has a high yield, preferably at a gram scale.

At least some of these goals could be met by the provision of a highly efficient synthesis route (overall yield nearly 28%) based on only four building blocks, three of which (compounds A, B and C) are reacted in a multicomponent Passerini reaction. The tubulysin derivatives thus obtained are super potent and can be attached towards biological matter (e.g. mAbs) through at least 3 different linker positions. Furthermore, the synthetic

method offers full stereo-control. The new approach allows to fine-tune the properties of tubulysin ADCs in a much better way than offered by current products.

Accordingly, the invention provides method for preparing a tubulysin derivative, comprising reacting compounds A, B and C in a 3-component Passerini reaction,

wherein compound A is a carboxylic acid according to the general formula A



wherein

R_1 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; or a substituted or unsubstituted benzyl,

R_2 represents H, a substituted or unsubstituted alkyl, or a substituted or unsubstituted cycloalkyl;

R_3 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; or a substituted or unsubstituted benzyl;

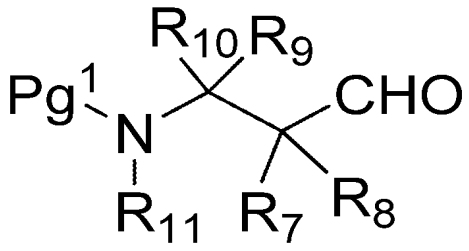
R_4 represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, a substituted or unsubstituted benzyl;

R_5 represents a substituted or unsubstituted alkyl or a substituted or unsubstituted cycloalkyl; preferably a substituted or unsubstituted cycloalkyl;

or wherein R_4 and R_5 are connected to form a 4 to 7 membered ring;

R_6 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; a substituted or unsubstituted benzyl, or COOR', where R' is an optionally substituted alkyl, cycloalkyl, benzyl or aroyl moiety;

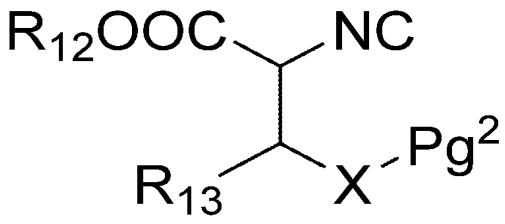
wherein compound B is an aldehyde according to the general formula B



wherein R_7, R_8, R_9, R_{10} and R_{11} each independently represent H, F, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl or a substituted or unsubstituted benzyl;

Pg^1 is an amine protecting group, preferably a carbamate, a substituted or an unsubstituted benzyl, or a substituted or an unsubstituted sulfonamide;

wherein compound C is an isocyanide according to the general formula C



wherein

R_{12} represents a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

R_{13} represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

X represents O, S, Se, or -NH-, preferably S; and

Pg^2 represents an X- protecting group, preferably selected from trityl, tert-butyl, adamantyl and substituted benzyl, more preferably trityl or tert-butyl.

Where it is indicated that a moiety may be substituted, such as by use of "unsubstituted or substituted" or "optionally substituted" phrasing as in "unsubstituted or substituted alkyl" or "optionally substituted benzyl," such moiety may have one or more independently selected substituents, preferably one to five in number, more preferably one or two in number. Substituents and substitution patterns can be selected by one of ordinary skill in the art, having regard for the moiety to which the substituent is attached, to provide compounds that are chemically stable and that can be synthesized by techniques known in the art as well as the methods set forth herein.

The term "substituents" refers to groups in which one or more hydrogen atoms are replaced with one or more moieties selected from the group consisting of hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate and phosphonate. One or more of the hydrogen atoms attached to carbon atom may be replaced by one or more halogen atoms, e.g. fluorine or chlorine or both, such as trifluoromethyl, difluoromethyl, fluorochloromethyl. In one embodiment, one or more hydrogen atoms is replaced by fluorine, chlorine, bromine or iodine atoms or OH, = O, SH, = S, NH_2, = NH or NO_2 groups. This expression further refers to groups which are exclusively or

additionally replaced with unsubstituted C_1-C_6 alkyl, C_2 -C_6 alkenyl, C_2 -C_6 alkynyl, C_1 -C_6 heteroalkyl, C_3 -C_{10} cycloalkyl, C_2 -C_9 heterocycloalkyl, C_6 -C_{10} aryl, C_1 -C_9 heteroaryl, C_7 -C_{12} aralkyl or C_2 -C_{11} heteroaralkyl groups.

In a specific aspect, the term "substituted alkyl", "substituted cycloalkyl" or "substituted benzyl" refers to an alkyl, cycloalkyl or benzyl that is substituted with 1-5 substituents, preferably 1 or 2 substituents, selected from the group consisting of hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, and phosphonate. In another specific aspect, one or more e.g. 1-5, 1-3 or 1, of the hydrogen atoms attached to carbon atom of the alkyl, cycloalkyl or benzyl is replaced by one or more halogen atoms, e.g. fluorine or chlorine or both, such as trifluoromethyl, difluoromethyl, fluorochloromethyl. Also encompassed are substituted alkyl, cycloalkyl and benzyl moieties wherein one or more e.g. 1-5, 1-3 or 1, hydrogen atom(s) is replaced by fluorine, chlorine, bromine or iodine atoms or OH, = O, SH, = S, NH_2, = NH or NO_2 groups.

The term "alkyl," as used herein, unless otherwise specified, refers to a saturated straight or branched hydrocarbon chain of typically C_1 to C_{10}, and specifically includes methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, isopentyl, neopentyl, hexyl, isohexyl, cyclohexyl, cyclohexylmethyl, 3-methylpentyl, 2,2-dimethylbutyl, and 2,3-dimethylbutyl, and the like.

In one embodiment, the alkyl is a lower alkyl. The term "lower alkyl," as used herein, and unless otherwise specified, refers to a C_1 to C_4 saturated straight or branched alkyl group, including both substituted and unsubstituted forms as defined above. Unless otherwise specifically stated in this application, when alkyl is a suitable moiety, lower alkyl is preferred. Similarly, when alkyl or lower alkyl is a suitable moiety, unsubstituted alkyl or lower alkyl is preferred.

Alkyl groups can be optionally substituted with one or more moieties selected from the group consisting of hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate. One or more of the hydrogen atoms attached to carbon atom on alkyl may be replaces by one or more halogen atoms, e.g. fluorine or chlorine or both, such as trifluoromethyl, difluoromethyl, fluorochloromethyl.

The term "cycloalkyl", as used herein, refers to a saturated or partially unsaturated (e.g. cycloalkenyl) cyclic group containing one or more rings (preferably 1 or 2), the total of 3 to 14 ring carbon atoms, preferably 3 to 10 (especially 3 containing 4, 5, 6 or 7) ring carbon atoms. In one embodiment, it refers to a saturated hydrocarbon ring having 3-8 carbon atoms, preferably, 3-6 carbon atoms, such as cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl. The cycloalkyl group may also be substituted on the ring by and alkyl group, such as cyclopropylmethyl and the like.

In one embodiment, the term "substituted benzyl" refers to a benzyl radical which is substituted by fluorine, chlorine, bromine, nitro, C_1-C_4 alkyl, C_1-C_4-halogenoalkyl or C_1-C_4 alkoxy. For example, the benzyl is substituted with chlorine, bromine, nitro, methyl or methoxy.

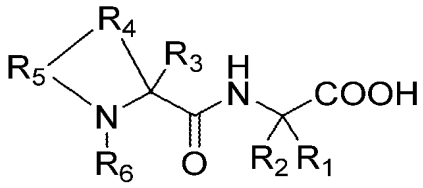
Unless particular stereoisomers are specifically indicated (e.g., by a bolded or dashed bond at a relevant stereocenter in a structural formula, by depiction of a double bond as having E or Z configuration in a structural formula, or by use stereochemistry-designating nomenclature), all stereoisomers are included within the scope of the invention, as pure compounds as well as mixtures thereof. Unless otherwise indicated, individual enantiomers, diastereomers, geometrical isomers, and combinations and mixtures thereof are all encompassed.

As will be understood, any combination of preferred or specifically disclosed compounds A, B and C may be used in a method of the invention.

In one embodiment, the invention uses a carboxylic acid compound A of the general formula A, wherein R_1 represents isopropyl, tert-butyl, iso-butyl, sec-butyl, cyclopropylmethyl or cyclobutylmethyl;R_2 is H; R_3 is $-(CH_2)n-CH_3$, wherein n is 3, 4 or 5; R_4 and R_5 are connected to form a 4 to 7 membered ring; or R_5 is a substituted or unsubstituted cycloalkyl; and/or R_6 is selected from the group consisting of benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate and ethyl carbamate.

In a specific aspect, compound A is of the formula A' wherein R_4 and R_5 are connected, and together form a cyclic structure comprising 3, 4, 5, 6 or 7 carbon atoms.

Formula A'



In one embodiment, R_3 represents H or methyl, and /or R_6 represents H, methyl, ethyl, propyl or cyclopropyl.

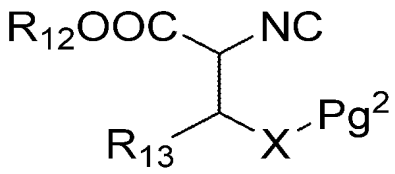
In a preferred embodiment, the method uses an aldehyde compound B according to the general formula B wherein R_7 is H; R_8 is H; R_9 is selected from the group consisting of isopropyl, cyclopropyl, cyclobutyl, isobutyl, sec-

butyl, tert-butyl and cyclopropylmethyl; R_{10} is H; and/or R_{11} is selected from the group consisting of methyl, ethyl, propyl, butyl, isopropyl, cyclopropyl and cyclopropylmethyl.

The Pg^1 moiety in compound B is an amine protecting group, preferably a carbamate, a substituted or an unsubstituted benzyl. For example, Pg^1 is selected from the group consisting of benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate, ethyl carbamate, benzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl.

Preferred isocyanide compounds C according to the general formula C include those wherein R_{12} is methyl, ethyl or tert-butyl; R_{13} is H or methyl, and/or wherein X represents S. The X-protecting group Pg^2 is preferably selected from trityl, tert-butyl, adamantyl and substituted benzyl, more preferably Pg^2 is trityl or tert-butyl.

An isocyanide compound according to the general formula C wherein X is S has been disclosed in Vishwanatha et al. (J. Org. Chem. 2017, 82, 9585-9594). The invention provides an isocyanide compound (suitably derived from cysteine) according to the general formula C



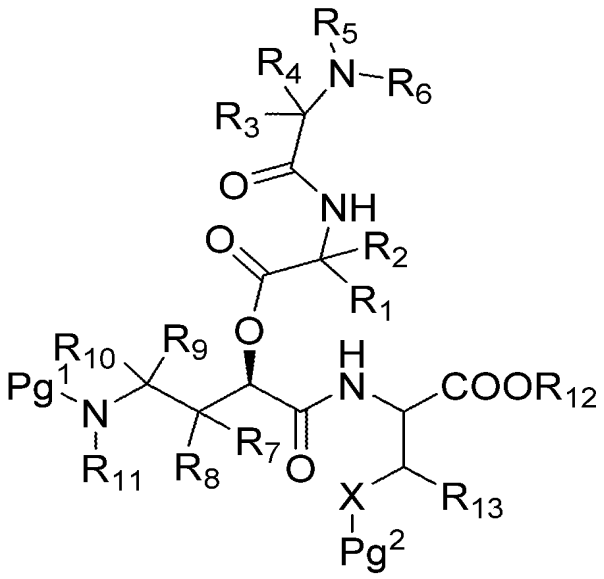
wherein the substituents are as defined herein above, and the use of the compound in the manufacture of a tubulysin derivate, preferably involving a 3-component Passerini reaction as herein disclosed.

Any one of the starting compounds for use in a method of the invention can be readily synthesized using conventional organic synthesis routes. Exemplary synthetic routes for exemplary compounds A, B and C are provided in the Examples.

In a method of the invention, compounds A, B and C can be reacted in any suitable solvent or solvent mixture. In one embodiment, a non-coordinating solvent or solvent mixture is used. For example, good results can be obtained with CH_2Cl_2, $CHCl_3$, CCl_4, benzene, THF, CH_3CN, 1,4-dioxane, or 1,2-dichloroethane. In a specific aspect, compounds A, B and C are reacted in a mixture of CH_2Cl_2:THF (1:1 v/v). The reaction is most easily performed at room temperature. However, reaction conditions above or below room temperature are also encompassed. The reaction time can range from a few hours up to a few days. Typically, it ranges from about 16 to 60 h, like 24 to 48 h. The three starting compounds may be present in the starting reaction mixture about equimolar amounts, for example wherein A : B : C is 0.8-1.2: 0.8-1.2: 0.8-1.2, preferably 0.9-1.1:0.9-1.1:09-1.1. The Passerini reaction is preferably carried out from 1 mmol to 100 mmol scale.

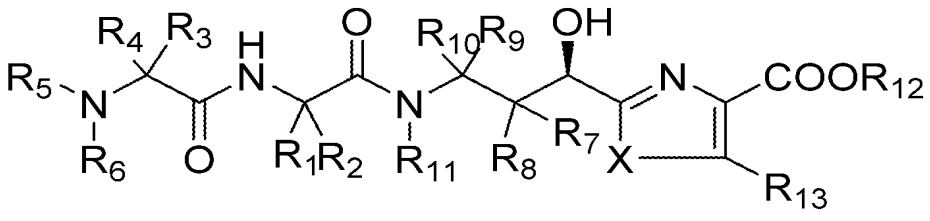
After completion of the Passerini reaction, the solvent(s) may be evaporated under reduced pressure. The reaction products are suitably purified through flash chromatography (e.g. cyclohexane/EtOAc 20:80 v/v). The by-product can also be recovered and coupled with dipeptide acid to form an ester.

Hence, in one embodiment a method of the invention further comprising isolating the 3-component Passerini reaction product of Formula D



wherein each of the (preferred) substituents is as defined herein above.

In order to obtain a tubulysin derivative, a method of the invention advantageously further comprises subjecting the Passerini reaction product of formula D to (a) an acyl migration reaction and (b) a cyclodehydration reaction of the Cys-amide. This will yield a thiazole compound of the general formula E having a hydroxyl moiety



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wherein each of the (preferred) substituents is as defined herein above.

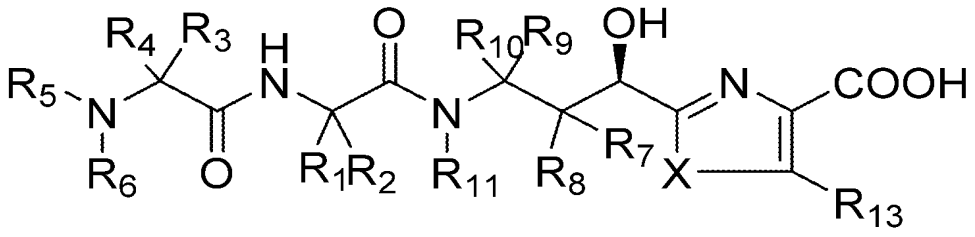
The acyl migration reaction is suitably performed in a two-step process involving exposure to diethylamine (DEA) followed by exposure to trimethylamine (TEA) using slight modification of existing methods. See for

example Fauré et al.(Org. Lett. 2009, 11(5), pp 1167-1170) disclosing conditions for Amine Deprotection–Acyl Migration.

Cyclodehydration of the Cys-amide may also be performed in a two-step process, preferably involving incubation in the presence of $TiCl_4$ to obtain a thiazoline-containing compound, followed by oxidation in the presence of MnO_2 to obtain a thiazole moiety. Activated MnO_2 having a pore size of ≤ 5 microns is preferred.

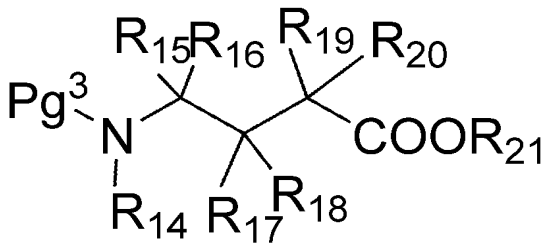
The order in which the acyl migration reaction and the cyclodehydration reaction are performed are not critical. In one embodiment, acyl migration precedes cyclodehydration. In another embodiment, cyclodehydration precedes acyl migration. Preferably, the method comprises cyclcodehydration, then oxidation followed by Fmoc deprotection and acyl migration.

As a next step, a method according to the invention advantageously further comprises hydrolyzing the R_{12}-containing ester of the general formula E to obtain a carboxylic acid compound of the general formula F

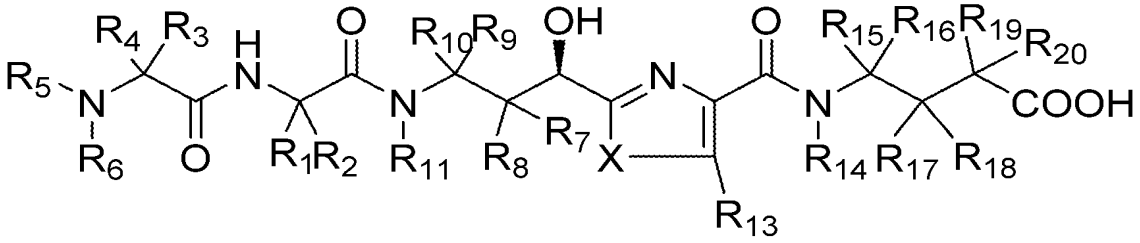


wherein each of the (preferred) substituents is as defined herein above.

Still further, the carboxylic acid compound of the formula F may be reacted with a compound of the general formula G



followed by removal of protecting moiety R_{21}, to obtain a compound of the general formula H



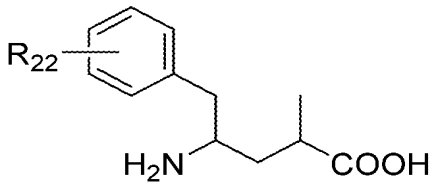
wherein R_{14}, R_{15}, R_{16}, R_{20} and protecting moiety R_{21} each independently represent H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl, R_{17} and R_{18} each independently represent H, F, a substituted or unsubstituted alkyl, or a substituted or unsubstituted cycloalkyl and R_{19} represents H, F, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl.

Pg^3 of compound G is an amine protecting group, preferably a carbamate, a substituted or unsubstituted benzyl, preferably selected from the group consisting of tertbutyl sulfine, benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl

carbamate, ethyl carbamate, benzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl.

In a preferred embodiment, R_{14} represents H;
R_{15} represents H, benzyl, (substituted) benzyl;
R_{16} represents H, benzyl, substituted benzyl (if R_{15} is H) and/or
R_{17} and R_{18} represent H or F.
R_{19} and/or R_{20} are preferably independently selected from the group consisting of methyl, ethyl, propyl, butyl, isopropyl, cyclopropyl and cyclopropylmethyl.
R_{21} is preferably H, methyl or ethyl.

In a specific embodiment, the carboxylic acid compound of the formula F is reacted with tubuphenylalanine of the formula



or a salt thereof, such as the Na, K, Li or Ca salt,
wherein R_{22} is H, OH, F or NO_2, preferably H, OH or F.

A further aspect of the invention relates to a method for providing a tubuphenylalanine of the above formula using (S)-(-)methylsuccinic anhydride as intermediary compound. This method typically only comprising 6 steps, is shorter than the current synthetic procedures used and moreover has a better yield (up to nearly 60%) and increased stereoselectivity.

In one embodiment, the method comprises the steps of :

(i) refluxing (S)-(-)methylsuccinic acid in the presence of acetyl chloride to obtain(S)-(-)methylsuccinic anhydride

(ii) opening of the succinic anhydride in the presence of. (substituted) benzyl magnesium chloride and Cu I to obtain a keto-pentanoic acid

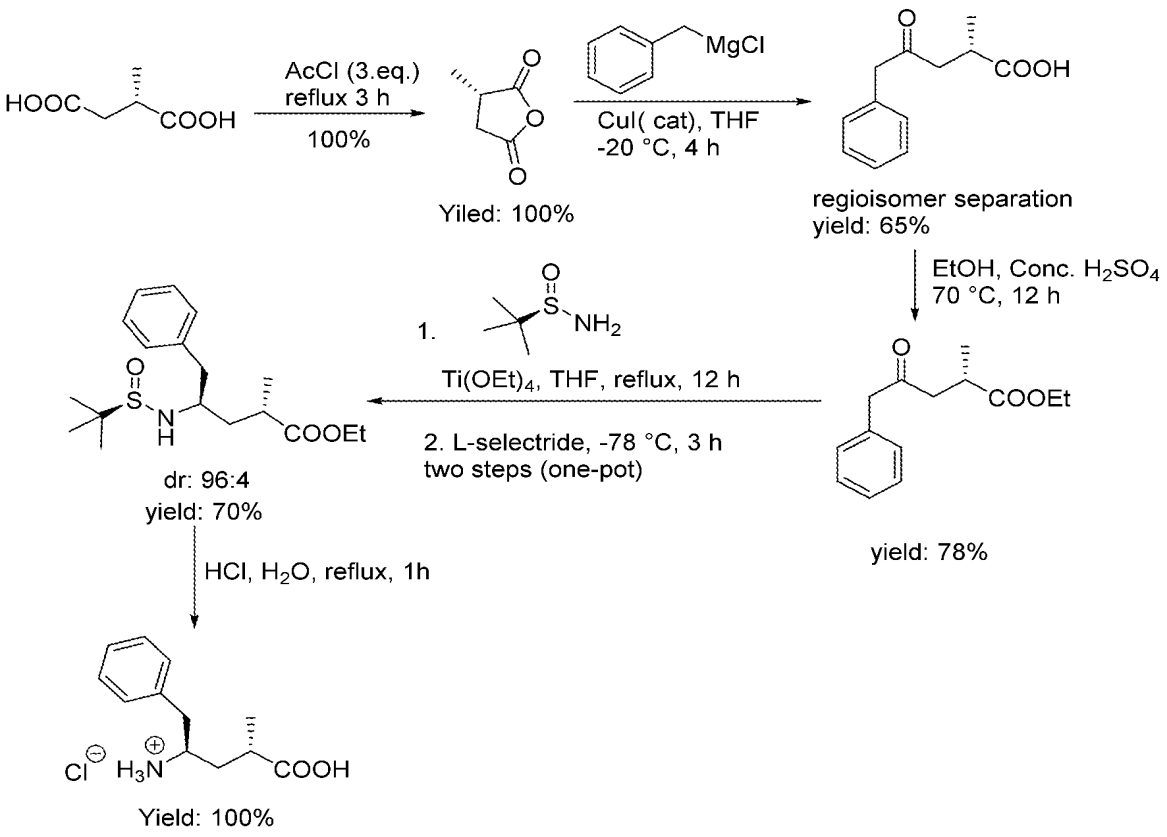
(iii) dissolving the keto-pentanoic acid in an alcoholic solvent and refluxing in the presence of a strong acid to yield the corresponding keto-pentanoic ester as oil;

(iv) reacting the keto-pentanoic ester with ((S)-*tert*-butylsulfinyl)amine in the presence of $Ti(OEt)_4$

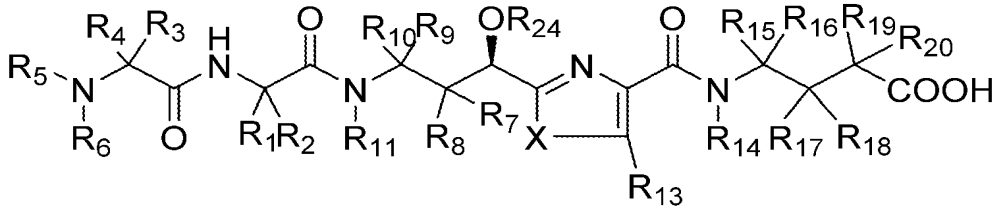
(v) cooling the reaction mixture of (iv) to a temperature below about -75°C; and

(vi) adding L-selectride in a dropwise fashion to obtain (2*S*, 4*R*)-4-(((*S*)-tert-butylsulfinyl)amino)-2- methyl-5-(substituted)phenylpentanoate.

An exemplary method for providing a tubuphenylalanine compound is depicted in the following scheme:



A method of the invention for providing a tubulysin derivative may further comprise the acylation of the hydroxyl group a compound of the general formula H to obtain an ac(et)ylated compound of the general Formula I

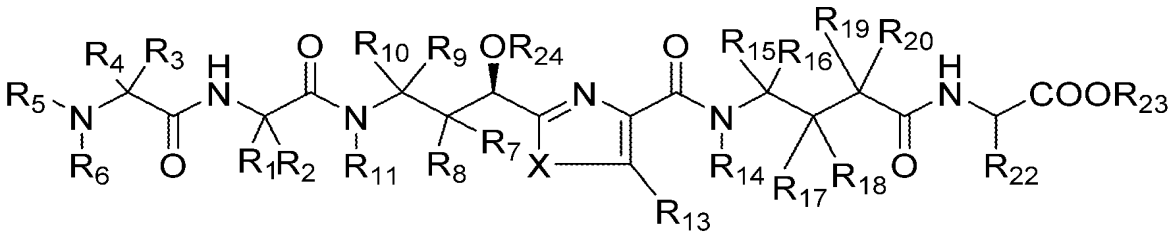


wherein R_{24} represent acetyl, acyl (substituted) alkyl, acyl cycloalkyl, or acyl benzyl, preferably acetyl or acyl derivative of methyl, ethyl, tert-butyl or benzyl.

A method of the present invention allows for the introduction of a variety of different linker types used in the conjugation of payloads to small molecules, polymers, peptides, proteins, antibodies, antibody fragments etc. can be adopted and thereby, many different conjugation methods can be applied. Spacer systems at different positions can be used either directly for conjugation by using different conjugation technologies such as chemical conjugation methods known in the art, or enzymatic conjugations using transglutaminases, sortases or other enzymes or which can be used in combination with commonly described linker systems known in the art . More in particular, a method as herein disclosed provides tubulysin derivatives that can be attached toward biological matter, e.g. monoclonal antibodies, through at least 3 different linker positions. Herewith, the properties of, for example, ADC's can be fine-tuned in a better way as compared to existing products.

Therefore, the present invention particularly finds it use in the manufacture of a cytotoxic tubulysin derivative or tubulysin prodrug.
In one embodiment, a method as herein disclosed provides a conjugate comprising a tubulysin compound covalently linked to a targeting moiety that specifically or preferentially binds to a chemical entity on a target cell, which target cell preferably is a cancer cell. Preferably, the targeting moiety is an antibody—more preferably a monoclonal antibody; even more preferably a human monoclonal antibody—and the chemical entity is a tumor associated antigen. The tumor associated antigen can be one that is displayed on the surface of a cancer cell or one that is secreted by a cancer cell into the surrounding extracellular space.

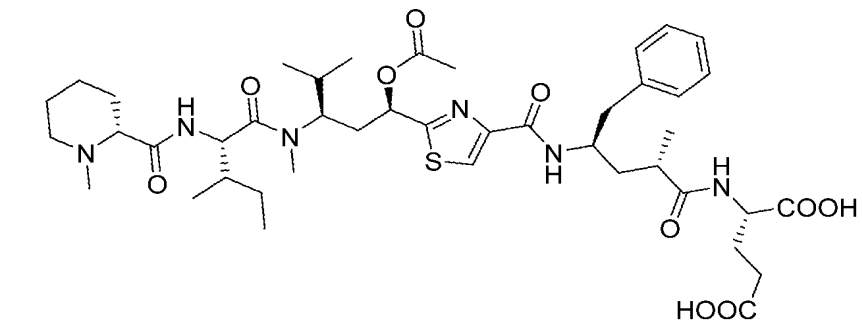
In one aspect, a method of the invention comprising a step of reacting the ac(et)ylated compound of the general Formula I with Glu-$(OMe)_2$ or alternative amino acid ester to obtain a compound of the formula J

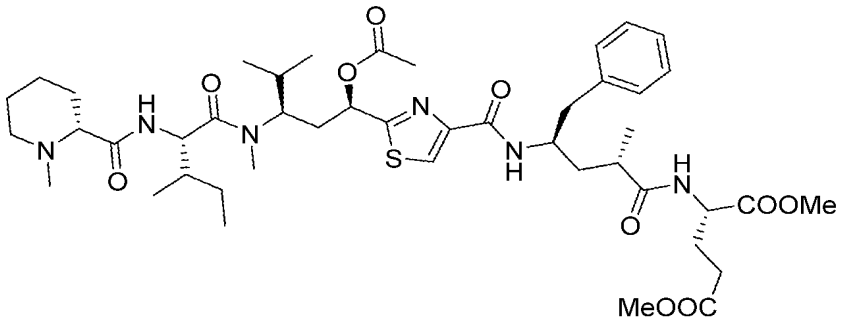


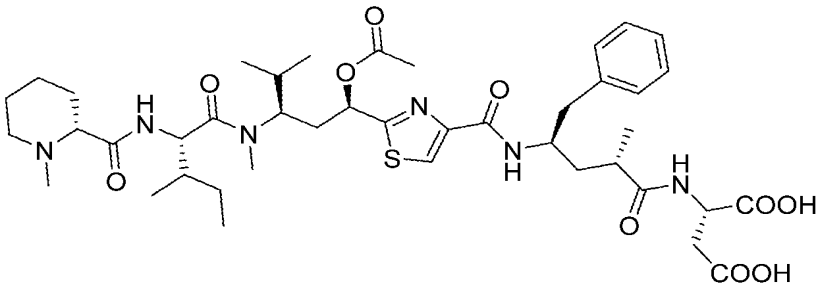
wherein R_{22} is H or an amino acid side chain, preferably CH_2-CH_2-COOZ wherein Z is H or methyl,

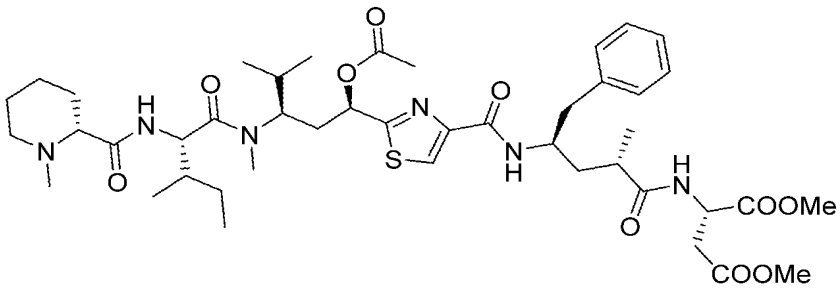
R_{23} = H or a carboxyl protecting group, in particular methyl, ethyl, or tert-butyl; preferably H, methyl, ethyl or tert-butyl.

In a specific aspect, the method comprises the synthesis of a glutamic acid conjugate of tubulysin, which finds its use in a prodrug approach using glucarpidase or an enzyme showing a similar activity. For example, the method comprises the formation of a tubulysin-prodrug comprising one or more glutamate residue(s) linked to an amidic, urethanic or ureidic bond. For example, the invention provides one of the following exemplary compounds



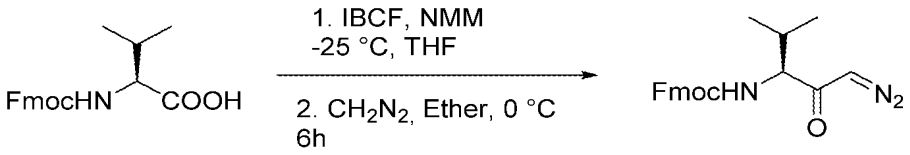






EXPERIMENTAL SECTION

Example 1: Preparation of N$^{\alpha}$-Fmoc-valine diazoketone



1 2

Fmoc-Val-OH (1.63 g, 4.79mmol) was dissolved in anhydrous THF (10mL) under nitrogen at 25 °C. N-Methylmorpholine (0.55mL, 5mmol) and isobutylchloroformate were then added to the mixture and after 5min of stirring, the mixture was cooled down to 78 °C. Anhydrous ether (10mL) was added and the fine suspension was filtered under N_2. Diazomethane (294mg, 7mmol) was added dropwise and the reaction was allowed to stir for 2 h at room temperature. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2, and washed with water (3 X 10mL) before drying over $MgSO_4$ and concentration. Fmoc-Val-CHN_2 were purified via flash chromatography using EtOAc/hexane (30/70, v/v).

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 95%

$[\alpha]_D^{25}$ = +16.3 (*c* 1, $CHCl_3$)

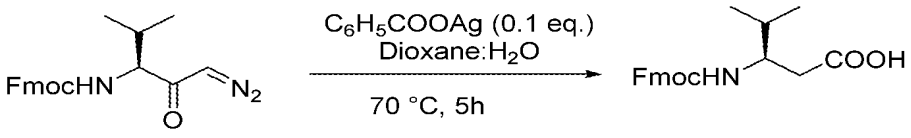
MS (ESI) m/z calculated for $C_{21}H_{21}N_3O_3$ $[M+Na]^+$: 386.14, found 386.10

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.43 -6.91 (8H, m), 6.24 (br, s, 1H), 5.07 (d, *J* = 6.9 Hz, 1H), 4.70 (d, *J* = 2.0 Hz, 2H), 4.48 – 4.43 (m, 1H), 2.54 (h, *J* = 6.8 Hz, 1H), 0.93 (dd, *J* = 24.9, 6.8 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$): 191.2, 158.6, 144.2, 139.6, 126.9, 126.8, 123.8, 122.7, 67.0, 65.9, 60.6, 48.4, 31.2, 19.4.

Ref: Tetrahedron Letters 45 (2004) 8603–8606.

Example 2: Synthesis of Fmoc-β-Val-OH



2 **3**

A solution of **2** (1.0 mmol) in 1, 4-dioxane (10 mL) and water (5 mL) was treated with silver benzoate (0.02 mmol). It was refluxed at 70 °C for 5 h and then filtered. The solvent was evaporated under reduced pressure. The residue was dissolved in aqueous sodium carbonate (10%, 20 mL) and washed with diethyl ether (2 X 30 mL). The aqueous layer was acidified to pH 2–3 (using 2N HCl) and extracted with ethyl acetate (3 X 25 mL). The combined organic layer was washed with water and dried over anhydrous sodium sulphate and evaporated. The residue was recrystallized using ethyl acetate and *n*-hexane to give **3**. (95% yield).

TLC: 0.3 (CH_2Cl_2/ MeOH, 10:.0.5),

Yield: 98%

$[\alpha]_D^{25}$ = + 25.5 (*c* 1, $CHCl_3$)

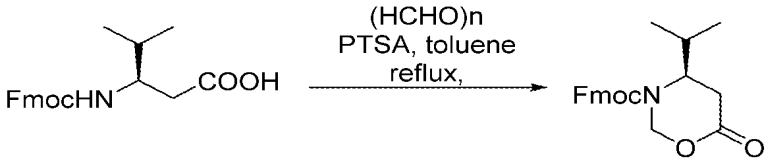
MS (ESI) m/z calculated for $C_{21}H_{23}NO_4$ $[M+Na]^+$: 376.15, found 376.08

^1H NMR (500 MHz, $CDCl_3$): δ 7.79- 7.43 (m, 8 H), 4.94 (s, 1H), 4.70 (d, J = 5.6 Hz, 2H), 4.43 (q, J = 7.0 Hz, 1H), 4.32 – 4.25 (m, 1H), 2.79 (dd, J = 12.4, 7.0 Hz, 1H), 2.67 – 2.53 (m, 2H), 0.81 (dd, J = 25.1, 6.8 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$): 174.4, 158.5, 144.2, 139.6, 126.9, 126.8, 123.8, 122.7, 67.0, 58.9, 48.4, 34.6, 33.6, 19.1.

Ref: Syn. Commun., 2003, 33, 3089–3096.

Example 3: Synthesis of oxazolidinone 4



3 4

Fmoc-ß-Val **3** (1.0 mmol) was suspended in 20 mL of toluene, and paraformaldehyde (1 g) and p-toluenesulfonic acid (100 mg) were added. The mixture was refluxed for 30 min with azeotropic water removal. The solution was cooled, washed with 1 N aqueous $NaHC0_3$ (2 X 25 mL) and dried over Na_2S0_4. Concentration in vacuo to yield **4** as yellow gum (yield: 88%).

TLC: 0.38 (Petroleum ether/EtOAc, 7:3)

Yield: 80%

$[\alpha]_D^{25}$ = -15.9 (*c* 1, $CHCl_3$)

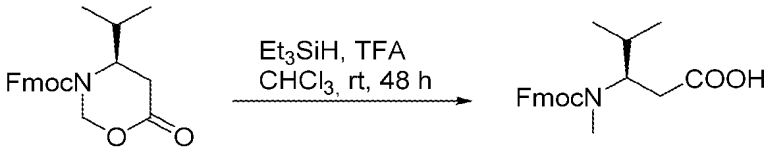
MS (ESI) m/z calculated for $C_{21}H_{23}NO_4$ $[M+Na]^+$: 388,15, found 388.10.

Major rotamer: ^1H NMR (500 MHz, $CDCl_3$): δ 8.10-7.44 (m, 8 H), 6.28 (d, J = 11.5 Hz, 1H), 6.20 (d, J = 11.5 Hz, 1H), 4.81 (q, J = 7.0 Hz, 1H), 4.70 (d, J = 5.7 Hz, 2H), 4.36 – 4.29 (m, 1H), 2.69 – 2.53 (m, 2H), 2.46 (dd, J = 14.6, 7.0 Hz, 1H), 0.83 (dd, J = 25.1, 6.8 Hz, 6H).

Major rotamer: ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.8, 158.3, 144.8, 139.1, 127.0, 126.8, 123.8, 122.7, 73.2, 67.4, 52.2, 47.9, 38.2, 32.1, 19.1.

Ref: *J. Org. Chem.*, 1983, 48, 77-81.

Example 5: Synthesis of N-methyl-Fmoc-β-Val-OH 5



4 5

The oxazolidinone **4** (3.0 mmol) was dissolved in 15 mL of $CHCl_3$, and 15 mL of trifluoroacetic acid and triethylsilane (1.43 mL, 1.04 g, 9.0 mmol) were added. The solution was stirred at room temperature for 48 h followed by concentration in vacuo to an oil. The oil was dissolved in CH_2Cl_2 and reconcentrated several times. The resultant oil was purified on silica gel flash column chromatography (PE/ EA 100:0 to 20:80).

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 85%

$[\alpha]_D^{25}$ = -32.5 (*c* 1, $CHCl_3$)

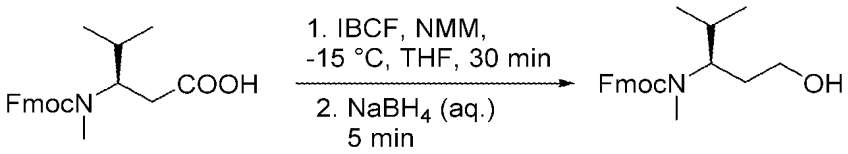
MS (ESI) m/z calculated for $C_{22}H_{25}NO_4$ $[M+Na]^+$: 390.16, 390.10.

Major rotamer: ^1H NMR (500 MHz, $CDCl_3$): δ 8.25-7.43 (m, 8H), 4.70 (d, J = 4.9 Hz, 2H), 4.16 (q, J = 7.0 Hz, 1H), 3.09 (s, 3H), 2.65 – 2.50 (m, 2H), 2.36 (dd, J = 12.4, 7.0 Hz, 1H), 0.88 (dd, J = 25.0, 6.8 Hz, 6H).

Major rotamer: ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.0, 156.0, 144.8, 139.1, 127.0, 126.8, 123.8, 122.7, 67.4, 53.6, 47.9, 34.2, 31.2, 19.1.

Ref: *J. Org. Chem.*, 1983, 48, 77-81.

Example 6: Synthesis of 6



5 **6**

A solution of **5** (25 mmol) in THF (50 mL) was cooled to -15 °C (ice/ salt bath) under a nitrogen atmosphere. NMM (25 mmol, 2.78 mL, 1 equiv) and IBCF (25 mmol, 3.40 mL, 1 equiv) were added successively in a dropwise manner. After 30 h, the reaction mixture was filtered. The filtrate was cooled to -15 °C (ice/ salt bath), and a solution of $NaBH_4$ (37.5 mmol, 1.42 g, 1.5 equiv) in H_2O (12.5 mL) was added in one portion. After complete reduction (TLC analysis), the suspension was diluted with EtOAc and H_2O

(1:1). The organic layer was separated and the aqueous layer was extracted with EtOAc (2X 25 mL) and the combined organic layer was dried over Na_2SO_4 and evoprated under vaccum to yield **6** as gummy solid.

TLC: 0.25 (Petroleum ether/EtOAc, 5:5)

Yield: 96%

$[\alpha]_D^{25}$ = +7.6 (*c* 1, $CHCl_3$)

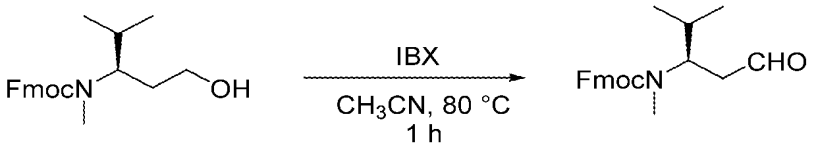
MS (ESI) m/z calculated for $C_{22}H_{27}NO_3$ $[M+Na]^+$: 376.18 (M + Na), found 376.08

Major rotamer: ^1H NMR (500 MHz, $CDCl_3$): δ 8.10-7.15 (m, 8 H), 4.70 (d, *J* = 5.2 Hz, 2H), 3.84 – 3.73 (m, 1H), 3.69 – 3.57 (m, 2H), 3.11 (s, 3H), 2.67 – 2.53 (m, *J* = 6.8 Hz, 1H), 2.16 – 2.01 (m, 2H), 1.41 (t, *J* = 5.5 Hz, 1H), 0.90 (dd, *J* = 25.1, 6.8 Hz, 6H).

Major rotamer: ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.0, 144.8, 139.1, 127.0, 126.8, 123.8, 122.7, 67.4, 59.5, 55.2, 47.9, 32.8, 31.2, 30.4, 19.1.

Ref: **J. Org. Chem.,** 2001, 66, 8454-8462

Example 7: Synthesis of Aldehyde (Exemplary compound B)



6 **7**

Alcohol **6** (1.00 mmol) was dissolved in CH_3CN (7 mL) and IBX (2.00 mmol) was added. The resulting suspension was immersed in an oil bath set to 80 °C and stirred vigorously open to the atmosphere. After 2 h (TLC monitoring), the reaction was cooled to room temperature and filtered through a medium glass frit. The filter cake was washed with 3 × 2 mL of ethyl acetate, and the combined filtrates were concentrated and purified by flash column chromatography to yield aldehyde **7** as a yellow gum.

TLC: 0.55 (Petroleum ether/EtOAc, 8:2)

Yield: 94%

$[\alpha]_D^{25}$ = +11.4 (*c* 1, $CHCl_3$)

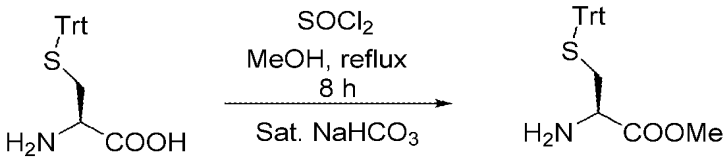
MS (ESI) m/z calculated for $C_{22}H_{25}NO_3$ $[M+Na]^+$: 374.17, found 374.10

Major rotamer: ^1H NMR (500 MHz, $CDCl_3$): δ 9.76 (t, *J* = 6.2 Hz, 1H), 8.10-7.00 (m, 8H), 4.70 (d, *J* = 5.3 Hz, 2H), 4.33 – 4.27 (m, 1H), 3.92 (q, *J* = 7.0 Hz, 1H), 3.18 (s, 3H), 2.83 (ddd, *J* = 12.3, 7.0, 6.1 Hz, 1H), 2.67 – 2.53 (m, 2H), 0.89 (dd, *J* = 25.0, 6.8 Hz, 6H).

Major rotamer: ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.5, 156.0, 144.8, 139.1, 127.0, 126.8, 123.8, 122.7, 67.4, 55.1, 47.9, 41.8, 31.2, 30.2, 19.1.

Ref: *Org. Lett.*, **2002**, *4*, 3001-3003.

Example 8: Synthesis of methyl S-trityl-L-cysteinate, 9



8 **9**

To a solution of S-trityl-L-cysteine (1.0 g, 2.76 mmol) in 50 mL of methanol stirred at 0 °C was added thionyl chloride (1.50 mL, 0.206 mmol) in a drop wise fashion. The solution was allowed to warm up to room temperature and then refluxed at 80 °C for 5 h. The solvent was removed under reduced pressure and the crude product was extracted with ethyl acetate and washed with saturated sodium bicarbonate for several times. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated to give ester as pale yellow gum.

TLC: 0.32 (Petroleum ether/EtOAc, 5:5)

Yield: 80%

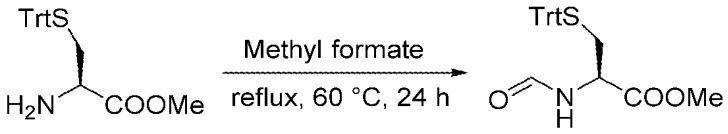
$[\alpha]_D^{25}$ = +20.4 (*c* 1, $CHCl_3$)

MS (ESI) m/z calculated for $C_{23}H_{23}NO_2S$ $[M+Na]^+$: 400.13, found 400.10

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.68 – 7.61 (m, 6H), 7.40 – 7.32 (m, 6H), 7.27 – 7.19 (m, 3H), 3.79 (t, J = 7.0 Hz, 1H), 3.71 (s, 3H), 3.64 (dd, J = 12.3, 7.0 Hz, 1H), 2.82 (dd, J = 12.4, 7.0 Hz, 1H), 2.19 (s, 2H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 172.0, 144.9, 129.0, 128.8, 128.4, 127.5, 126.5, 126.2, 67.4, 53.5, 52.4, 33.5.

Example 9: Synthesis of methyl N-formyl-S-trityl-L-cysteinate, 10



9 **10**

Amine **9** (1.0 g, 2.65 mmol) was dissolved in methyl formate (10 mL, solvent) and the assembly was allowed to reflux at 60 °C until TLC showed complete consumption of starting material (usually 24 h). The solvent was evaporated and the product was purified through column chromatography to yield formyl ester **10** as a white solid.

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 95%

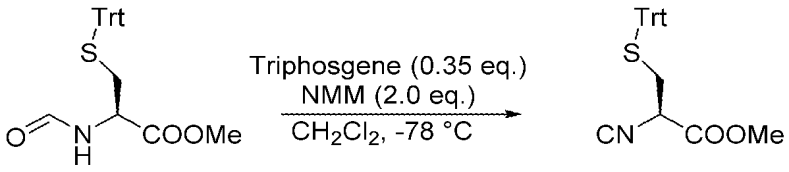
$[\alpha]_D^{25}$ = +9.6 (c 1, $CHCl_3$)

MS (ESI) m/z calculated for $C_{24}H_{23}NO_3S$ $[M+Na]^+$: 428.12, found 428.10

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.95 (d, J = 1.3 Hz, 1H), 7.50 – 7.11 (m, 16H), 6.14 (d, J = 8.1 Hz, 1H), 4.64 (dt, J = 8.2, 5.2 Hz, 1H), 3.68 (s, 3H), 2.77 (dd, J = 12.7, 5.8 Hz, 1H), 2.69 (dd, J = 12.9, 6.5 Hz, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.3, 160.4, 144.1, 129.4, 128.0, 128.0, 126.9, 126.8, 77.3, 77.1, 76.8, 67.0, 52.6, 49.7, 33.5.

Example 10: Synthesis of methyl (*R*)-2-isocyano-3-(tritylthio)propanoate (11) (Exemplary compound C)



10 **11**

Asolution of *N*-formyl Cys(Trt)-methyl ester (1.0 eq) in CH_2Cl_2 (5.0 mL), was cooled to -78 °C. N-methylmorpholine (2.0 eq) was added. After 5 min triphosgene (0.35 eq.) in CH_2Cl_2 (5.0 mL) was added drop wise and the reaction mixture was stirred for 3h at -78 ° C (TLC analysis). Saturated $NaHCO_3$ solution (10 mL) was added at same temperature and allowed to warm to room temperature. The reaction mixture was extracted with CH_2Cl_2, the organic extracts were separated, dried over anhydrous Na_2SO_4, filtered, and concentrated. The solution was diluted with diethyl ether (10 mL) and stored -15 °C for 5 h resulted in pure solid of isocyanide **11** which was collected by filtration.

TLC: 0.4 (Petroleum ether/EtOAc, 9:1)

Yield: 84%

$[\alpha]_D^{25}$ = +55.2 (*c* 1, $CHCl_3$)

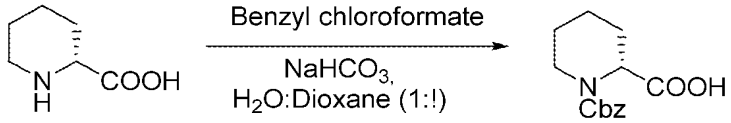
MS (ESI) m/z calculated for $C_{24}H_{21}NO_2S$ $[M+Na]^+$: 410.11, found 410.25

^1H NMR (500 MHz, $CDCl_3$): ^1H NMR (500 MHz, $CDCl_3$) δ 7.57 – 7.06 (m, 16H), 3.70 (s, 3H), 3.34 (ddd, *J* = 7.7, 5.8, 1.6 Hz, 1H), 2.89 – 2.63 (m, 2H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 165.6, 160.9, 143.9, 129.4, 129.2, 128.2, 128.0, 128.0, 127.9, 127.1, 67.5, 55.3, 53.4, 34.2.

Example 11: Synthesis of Acid component

11.1 Synthesis of Cbz-(D)-Pic-OH



12 **13**

D-picolinic acid (30 mmol) and $NaHCO_3$ (60.0 mmol) were dissolved in a solution of H2O and dioxane (1:1, 60 mL) was cooled to 0 °C. Benzylchloroformate (33.0 mmol) was added dropwise and the resulting suspension was stirred overnight at room temperature. The reaction mixture was quenched with 1N HCl till pH 2. This solution was extracted with EtOAc (3 X 15 mL) treated with brine and dried over Na_2SO_4. The solvent was evoprated under reduced pressure followed by recrystallization with diethyl ether afforded a white solid **13**.

TLC: 0.41 (Petroleum ether/EtOAc/TFA 5:5:0.1)

Yield: 95%

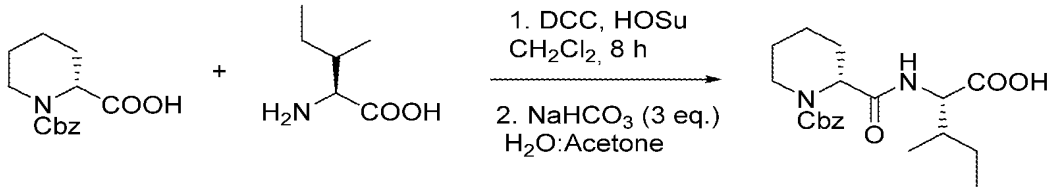
$[\alpha]_D^{25}$ = -155.2 (*c* 1, $CHCl_3$)

MS (ESI) m/z calculated for $C_{14}H_{17}NO_4$ $[M+Na]^+$: 286.10, found 286.18

Major Rotamer: ^1H NMR (500 MHz, $CDCl_3$): δ 7.38 – 7.27 (m, 5H), 5.83 (d, *J* = 12.4 Hz, 1H), 5.34 (d, *J* = 12.4 Hz, 1H), 5.05 (t, *J* = 6.9 Hz, 1H), 4.20 (dt, *J* = 12.5, 7.1 Hz, 1H), 3.32 (dt, *J* = 12.6, 7.1 Hz, 1H), 2.34 – 2.23 (m, 1H), 2.09 – 1.93 (m, 2H), 1.74 – 1.58 (m, 2H), 1.58 – 1.49 (m, 1H).

Major Rotamer: ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.7, 157.2, 137.1, 128.5, 128.0, 66.7, 55.3, 43.3, 26.3, 24.5, 20.7.

11.2 Synthesis of Dipeptide Acid (exemplary Compound A)



13 **14** **15**

A solution Cbz-picolinic acid (1.0 eq.) in anhydrous CH_2Cl_2 (10 mL) was cooled to 0 °C. N-hydroxysuccinimide (2.0 eq.) and DCC (1.2 eq.) were added. The reaction mixture was stirred at room temperature for 8h. The solution was filtered through pad of Cellite and the filtrate was evaporated under reduced pressure. In another flask Ile-OH (2 eq.) and $NaHCO_3$ (3.0 eq.) were dissolved in 15 mL of water. To this solution the succinamide ester in 20 mL of DMF were added dropwise over 10 min. The reaction mixture was stirred at room temperature for 2 h and then diluted with 20 mL of water. The reaction mixture was washed with diethyl ether (2 X 10 mL). The aqueous phase was acidified to pH 2 using 1N HCl and extracted with EtOAc (3 X 20 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ filtered and the solvent evaporated under reduced pressure to yield **15** (exemplary compound D).

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 79%

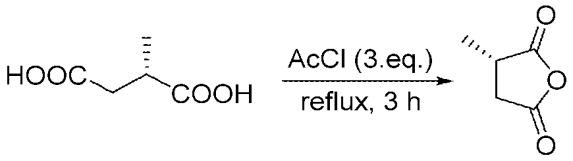
$[\alpha]_D^{25}$ = -65.6 (*c* 1, $CHCl_3$)

MS (ESI) m/z calculated for $C_{20}H_{28}N_2O_5$ $[M+H]^+$: 376.11, found 376.10

Major Rotamer: ^1H NMR (500 MHz, MeOD): δ 7.50 (s, 1H), 7.38 – 7.27 (m, 5H), 5.49 (d, *J* = 12.4 Hz, 1H), 5.37 (d, *J* = 12.4 Hz, 1H), 5.07 (t, *J* = 7.0 Hz, 1H), 4.43 – 4.33 (m, 2H), 3.53 (dt, *J* = 12.6, 7.1 Hz, 1H), 2.44 (dpd, *J* = 12.3, 6.9, 2.7 Hz, 1H), 2.15 – 1.96 (m, 2H), 1.82 – 1.55 (m, 3H), 1.53 – 1.28 (m, 3H), 1.01 (d, *J* = 6.8 Hz, 3H), 0.92 (t, *J* = 8.0 Hz, 3H).

Major Rotamer: ^{13}C NMR (100 MHz, MeOD): δ 175.2, 171.0, 157.2, 137.1, 128.5, 128.0, 66.7, 58.6, 55.6, 43.3, 36.1, 26.6, 24.5, 24.3, 20.8, 15.0, 11.6.

Example 12: Synthesis of (S)-(-)-methyl succinic anhydride



16 **17**

(S)-(-)-methyl succinic acid (10.0 mmol) and acetyl chloride (30.0 mmol) were placed in a single neck round bottomed flask and the assembly was refluxed on the steam bath for 3 h. The solution is allowed to cool undisturbed and is finally chilled in an ice bath. The succinic anhydride, which separates in beautiful crystals, is collected on a Büchner funnel, washed with two 75-cc. portions of cold ether, and dried in a vacuum desiccator.

TLC: 0.54 (Petroleum ether/EtOAc, 6:4)

Yield: 100%

$[\alpha]_D^{25}$ = -11.0 (*c* 1, $CHCl_3$)

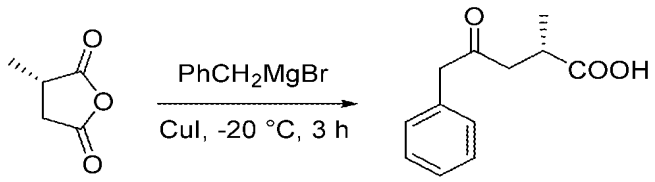
MS (ESI) m/z calculated for $C_5H_6O_3$ $[M+Na]^+$: 137.12, found 137.10

^1H NMR (500 MHz, $CDCl_3$): δ 3.48 (h, *J* = 6.9 Hz, 1H), 3.02 (dd, *J* = 18.2, 7.0 Hz, 1H), 2.56 (dd, *J* = 18.2, 7.0 Hz, 1H), 1.17 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 176.8, 172.9, 35.0, 30.3, 15.1.

Ref: *Org. Synth.* **1932**, *12*, 66

Example 13: Synthesis of (S)-2-methyl-4-oxo-5-phenylpentanoic acid, 18



17 **18**

To a solution of anhydride **17** (10 mmol) and CuI (0.1 mmol) in dry THF (50 mL) to -20 °C. benzyl magnesium bromide (2.0 M in THF, 12.0 mmol) slowly to the reaction mixture. (A purple color forms, but it disappear after the addition is finished). The reaction mixture was stirred at same temperature for additional 3h and then room temperature for 1h. H_2O (50 mL) and 1N HCl (50 mL) were added and stirred for 5 mins. The reaction mixture diluted with was EtOAc (50 mL X 2) and filtered through pad of Cellite. The organic layer was separated, dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was purified though column chromatography. The regioisomers were purified again to obtain desired product **18.**

TLC: 0.51 (Petroleum ether/EtOAc/TFA, 7:3:0.1)

Yield: 61%

$[\alpha]_D^{25}$ = -25.8 (*c* 1, $CHCl_3$)

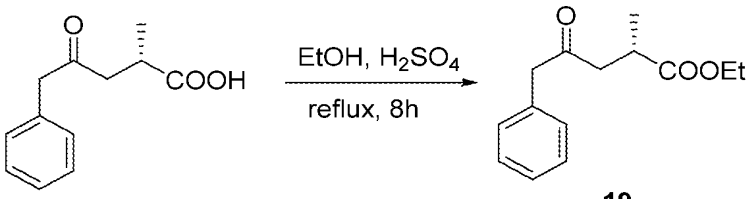
MS (ESI) m/z calculated for $C_{12}H_{14}O_3$ $[M+Na]^+$: 229.08, found 229.12

^1H NMR (500 MHz, $CDCl_3$): δ 7.38 – 7.28 (m, 4H), 7.28 – 7.20 (m, 1H), 3.71 (dt, *J* = 12.3, 1.0 Hz, 1H), 3.58 (d, *J* = 12.3 Hz, 1H), 2.98 (dd, *J* = 12.2, 7.0 Hz, 1H), 2.90 (dt, *J* = 13.6, 6.7 Hz, 1H), 2.61 (dd, *J* = 12.2, 6.8 Hz, 1H), 1.22 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 209.5, 178.1, 136.7, 129.5, 129.0, 126.8, 48.7, 45.6, 36.0, 17.9.

Ref: *J. Med. Chem.*, **2007**, *50* (18), pp 4261–4264

Example 14: Synthesis of ethyl (S)-2-methyl-4-oxo-5-phenylpentanoate, 19



18 19

The ketoacid **18** (7.0 mmol) was dissolved in EtOH (40 mL), concentrated H_2SO_4 (1.1 mL, 21.0 mmol) was added at room temperature, and then the reaction mixture was refluxed overnight. After evaporation of the solvent, H_2O (10 mL) was added, and the mixture was neutralized with a 2 M aqueous NaOH solution and extracted with EtOAc (3 × 15 mL). The combined organic phases were dried (Na_2SO_4). After filtration and evaporation of the solvent, the expected ketoester obtained as oil.

TLC: 0.45 (Petroleum ether/EtOAc, 9.5:0.5)

Yield: 90%

$[\alpha]_D^{25}$ = -20.6 (*c* 1, $CHCl_3$)

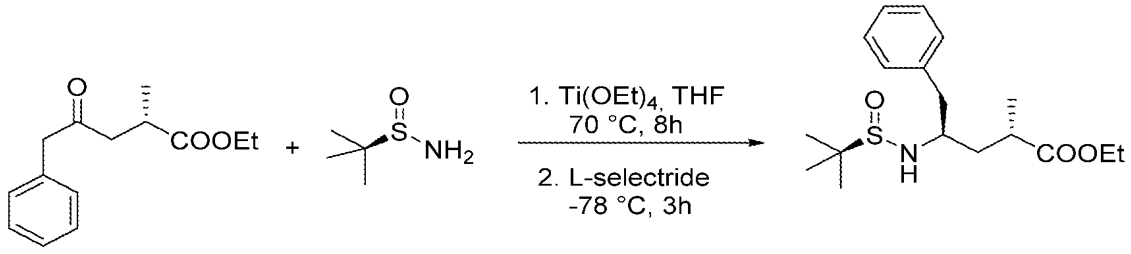
MS (ESI) m/z calculated for $C_{14}H_{18}O_3$ $[M+Na]^+$: 257.11, found 257.10

^1H NMR (500 MHz, $CDCl_3$): δ 7.38 – 7.27 (m, 4H), 7.27 – 7.20 (m, 1H), 4.68 (dq, *J* = 12.1, 5.9 Hz, 1H), 3.83 (dt, *J* = 12.4, 1.0 Hz, 1H), 3.60 (dq, *J* = 12.1, 6.0 Hz, 1H), 3.45 (d, *J* = 12.5 Hz, 1H), 3.35 (dd, *J* = 12.3, 7.0 Hz, 1H), 2.99 (h, *J* = 6.9 Hz, 1H), 2.46 (dd, *J* = 12.4, 7.0 Hz, 1H), 1.26 – 1.18 (m, 6H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 209.5, 176.6, 136.7, 129.5, 129.0, 126.8, 61.5, 48.7, 45.4, 36.0, 17.9, 14.1.

Ref: *J. Org. Chem.*, **2013**, *78*, 3647

Example 15: Synthesis of ethyl (2S,4R)-4-(((S)-*tert*-butylsulfinyl)amino)-2-methyl-5-phenylpentanoate, 21



19 **20** **21**

Ketone (1.0 mmol) was added to a solution of **20** (1.5 mmol) and $Ti(OEt)_4$ (3.0 mmol) in THF (15 mL) at room temperature (rt). The reaction mixture was heated at 70 °C for 8h and the reaction conversion was followed by TLC. Once the reaction was determined to be complete by TLC, the mixture was cooled to room temperature and then to -78 °C. L-Selectride (1 M solution in THF) was added dropwise. The reaction mixture was stirred at same temperature for 3 h. Once the reduction was determined to be complete by TLC, the reaction mixture was quenched by dropwise addition of MeOH at 0 °C until gas evolution was no longer observed. The crude reaction mixture was poured into an equal volume of brine while being rapidly stirred. The resulting suspension was filtered through a plug of Cellite, and the filter cake was washed with EtOAc. The filtrate was washed with brine, and the brine layer was extracted with EtOAc (3×). The combined organic portions were dried (Na_2SO_4), filtered, and concentrated. The product **21** was purified by silica gel chromatography (hexanes/EtOAc).

TLC: 0.42 (Petroleum ether/EtOAc, 6:4)

Yield: 89%

$[\alpha]_D^{25}$ = +6 (*c* 1, $CHCl_3$)

MS (ESI) m/z calculated for $C_{18}H_{29}NO_3S$ $[M+Na]^+$: 362.17, found 362.18

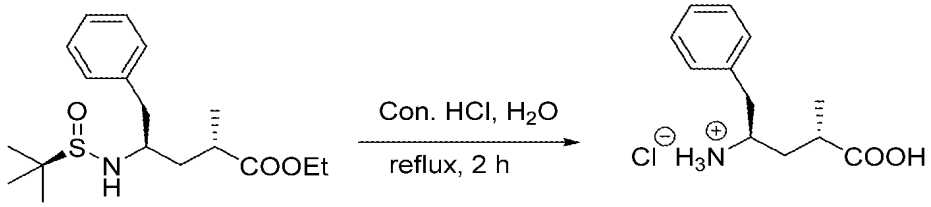
^1H NMR (500 MHz, $CDCl_3$): δ 7.32 – 7.24 (m, 2H), 7.24 – 7.13 (m, 3H), 4.78 (dq, *J* = 12.1, 6.0 Hz, 1H), 3.85 (s, 1H), 3.69 (dq, *J* = 12.2, 6.0 Hz, 1H), 3.60

(dtd, J = 11.9, 7.0, 2.2 Hz, 1H), 3.31 (ddt, J = 12.5, 6.9, 1.1 Hz, 1H), 2.92 (ddq, J = 12.3, 7.0, 0.8 Hz, 1H), 2.59 (h, J = 6.9 Hz, 1H), 2.28 (td, J = 12.1, 6.9 Hz, 1H), 2.13 – 2.05 (m, 1H), 1.29 – 1.20 (m, 15H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 177.6, 137.0, 129.3, 128.4, 126.3, 69.6, 61.5, 56.7, 39.9, 38.7, 37.7, 20.4, 18.1, 14.1.

Ref: *J. Org. Chem.*, **2007**, *72*, 626-629

Example 16: Synthesis of Tubuphenylalanine (22) (Exemplary compound G).



21 **22**

H_2O (3.07 mL) was added to **21** (1.0 mmol), followed by conc. HCl (3.07 mL), and the reaction mixture was heated to 100 °C for 2h. The mixture was then allowed to cool to room temperature and concentrated under vacuum. The resulting oil was triturated three times with Et_2O to give **22** (100%) as a white powder.

TLC: 0.31 (CH_2Cl_2/MeOH/NH_4OH, 9:1:0.2)

Yield: 95%$[\alpha]_D^{25}$ = +15.2 (c = 1, MeOH)

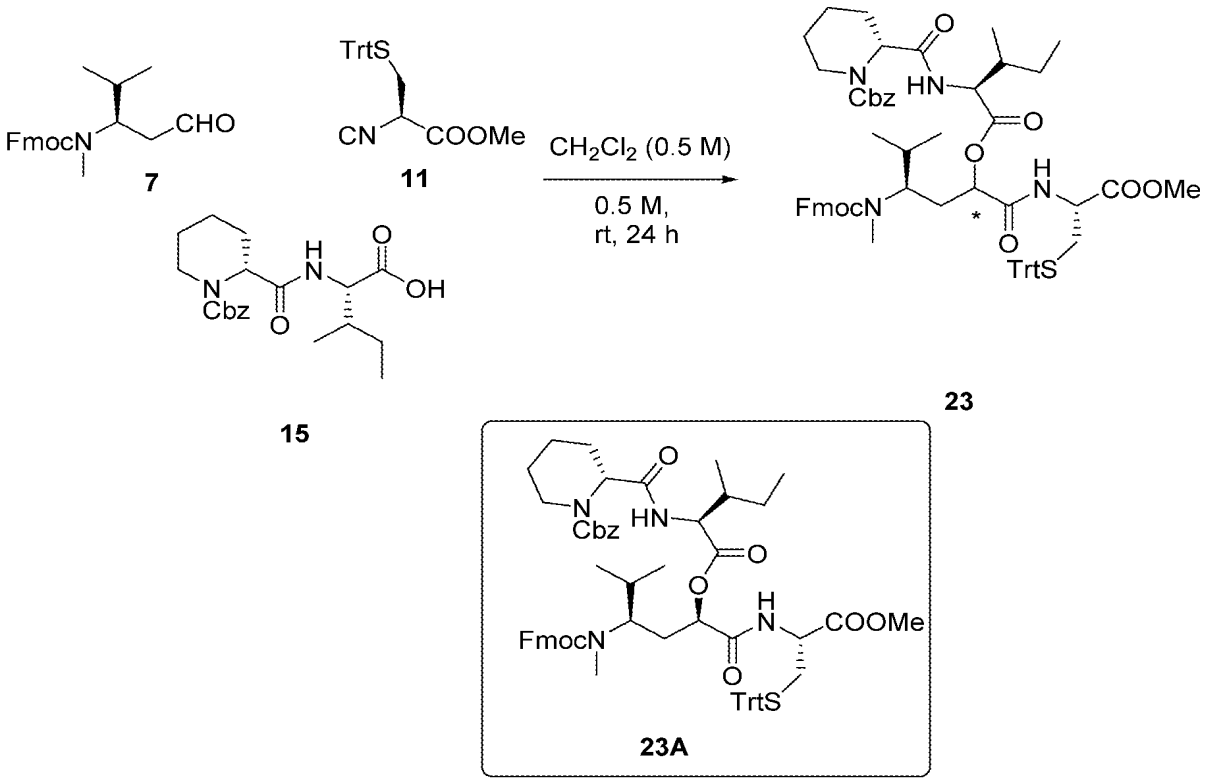
MS (ESI) m/z calculated for $C_{12}H_{17}NO_2$ $[M+H]^+$: 208.13, found 208.05

^{1}H NMR (500 MHz, D_2O) δ 7.29 (m, 2H), 7.20 (t, 1H, J = 7.3 Hz), 7.22 (d, 2H, J = 7.1 Hz), 3.49 (m, 1H), 2.85 (dd, 1H, J = 6.6, 14.2 Hz), 2.82 (dd, 1H, J = 7.6, 14.2 Hz), 2.40-2.60 (m, 1H), 1.77-1.88 (m, 1H), 1.42-1.68 (m, 1H), 1.06 (d, 3H, J = 7.0 Hz).

^{13}C NMR (125 MHz, D2O) δ 180.1, 135.2, 129.8, 128.8, 128.0, 51.6, 39.0, 36.5, 36.0, 17.0.

129.8, 130.1, 136.1, 180.7.

Example 17: Total Synthesis of 23



To a mixture of dipeptide acid **15** (1.0 eq), aldehyde **7** (1.0 eq) and isocyanide **11** (1.1 eq) in CH_2Cl_2 (2 mL) was stirred at room temperature for 24-48 h. The solvents were evaporated under reduced pressure and purified through flash chromatography (cyclohexane/EtOAc 20:80). The mixture of diastereomers **23** were purified again and pure diastereomer **23A** was used further.

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 80%

$[\alpha]_D^{25}$ = -96.5 (*C* 1, $CHCl_3$)

MS (ESI) m/z calculated for $C_{66}H_{74}N_4O_{10}S$ $[M+Na]^+$: 1137.2, found 1137.25

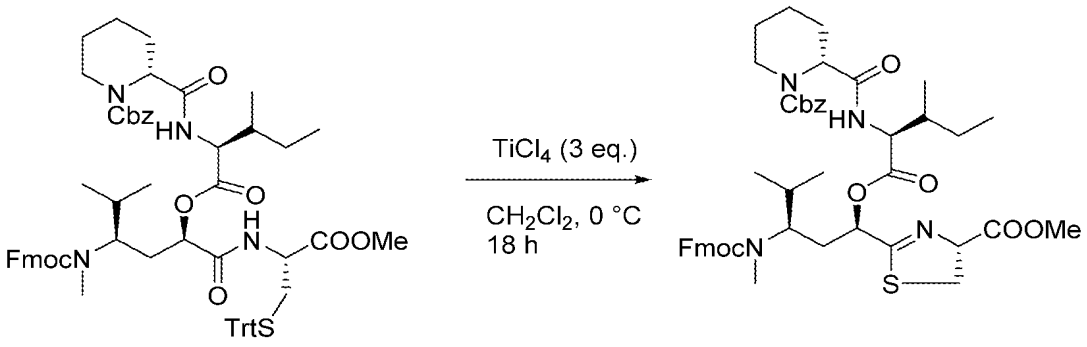
Major rotamer: ^1H NMR (500 MHz, $CDCl_3$): δ 7.93 – 7.84 (m, 3H), 7.82 (dt, *J* = 7.2, 1.8 Hz, 2H), 7.71 – 7.64 (m, 2H), 7.59 – 7.52 (m, 4H), 7.50 (s, 2H), 7.45 (tdd, *J* = 7.5, 6.0, 1.5 Hz, 2H), 7.42 – 7.13 (m, 15H), 5.01 (t, *J* = 7.0 Hz,

1H), 4.92 (d, J = 7.0 Hz, 1H), 4.82 – 4.72 (m, 2H), 4.72 – 4.67 (m, 2H), 4.49 (dt, J = 12.5, 6.9 Hz, 1H), 4.35 – 4.28 (m, 1H), 4.17 (ddd, J = 11.9, 7.0, 2.5 Hz, 1H), 3.71 (s, 3H), 3.51 – 3.41 (m, 1H), 3.33 (dd, J = 12.4, 7.0 Hz, 1H), 3.25 (s, 3H), 2.98 – 2.84 (m, 2H), 2.81 – 2.69 (m, 1H), 2.67 – 2.53 (m, J = 6.8 Hz, 1H), 2.07 – 1.91 (m, 2H), 1.91 – 1.44 (m, 6H), 1.02 – 0.85 (m, 12H).

Major rotamer: ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.0, 169.9, 157.2, 156.0, 144.9, 139.1, 137.1, 128.8, 128.6, 128.5, 128.0, 127.5, 127.0, 126.8, 123.8, 122.7, 69.5, 66.7, 59.8, 55.6, 53.9, 53.5, 52.4, 47.9, 43.3, 35.5, 33.2, 32.2, 26.6, 24.5, 24.3, 20.8, 19.1, 15.0, 11.6.

Ref: Org. Lett., **2009**, *11* (5), pp 1167–1170

Example 18: Synthesis of thiazoline, 24



23A **24**

A solution of S-trityl-protected cysteine N-amide **23A** (1.0 mmol) in dry CH_2Cl_2 (10 mL) was treated with $TiCl_4$ (1.0 M solution in CH_2Cl_2, 3.0 mmol) and stirred at 0 °C for 18 h. The reaction mixture was stirred vigorously with cold saturated aqueous $NaHCO_3$ (2×) for 30 min. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The resultant crude product was purified by flash chromatography to afford **24** as a yellow foam.

TLC: 0.32 (Petroleum ether/EtOAc, 6:4)

Yield: 75%

$[\alpha]_D^{25}$ = -108.3 (C 1, $CHCl_3$)

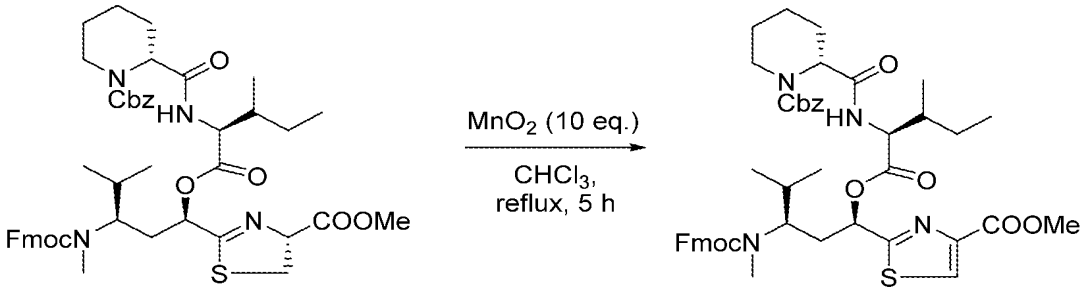
MS (ESI) m/z calculated for $C_{47}H_{58}N_4O_9S$ $[M+Na]^+$: 877.12, found 877.20

^1H NMR (500 MHz, $CDCl_3$): δ 7.85 – 7.76 (m, 2H), 7.59 – 7.48 (m, 4H), 7.44 (td, *J* = 7.5, 1.5 Hz, 1H), 7.40 – 7.27 (m, 6H), 5.06 (dt, *J* = 26.4, 7.1 Hz, 2H), 4.77 – 4.67 (m, 3H), 4.55 (t, *J* = 6.9 Hz, 1H), 4.42 (dt, *J* = 12.5, 7.1 Hz, 1H), 4.27 (td, *J* = 7.3, 0.8 Hz, 1H), 4.08 (ddd, *J* = 11.9, 7.0, 2.4 Hz, 1H), 3.85 (dd, *J* = 12.5, 7.0 Hz, 1H), 3.71 (s, 3H), 3.40 (dd, *J* = 12.5, 7.0 Hz, 1H), 3.22 (s, 3H), 3.03 (dt, *J* = 12.4, 7.1 Hz, 1H), 2.81 – 2.69 (m, 1H), 2.67 – 2.53 (m, *J* = 6.8 Hz, 1H), 2.17 (ddd, *J* = 12.4, 7.0, 2.4 Hz, 1H), 2.02 – 1.90 (m, 2H), 1.83 (dp, *J* = 13.0, 7.1 Hz, 1H), 1.76 – 1.48 (m, 3H), 1.42 (dp, *J* = 13.0, 7.1 Hz, 1H), 1.23 – 1.11 (m, 2H), 1.02 – 0.92 (m, 6H), 0.81 (d, *J* = 6.8 Hz, 3H), 0.72 (d, *J* = 6.8 Hz, 3H).

Major Rotamer: ^{13}C NMR (125 MHz, $CDCl_3$): 172.9, 172.1, 171.0, 170.1, 157.2, 156.0, 144.8, 139.1, 137.1, 128.5, 128.0, 127.0, 126.8, 123.8, 122.7, 74.0, 67.4, 66.7, 65.6, 59.8, 55.6, 53.5, 51.7, 47.9, 43.3, 35.8, 35.5, 34.3, 26.6, 24.5, 24.3, 20.8, 19.1, 15.0, 11.6.

Ref: Org. Lett., 2000, 2, 3289.

Example 19: Synthesis of thiazole, 25



24 **25**

To a solution of **24** (1 mmol) in $CHCl_3$ (5 mL), activated MnO_2 (< 10 micron, 10 mmol) was added. The reaction mixture was refluxed at 70 °C for 5 h, then filtered through a short silica gel and celite column and washed with $CHCl_3$. The organic solution was concentrated. The resulting crude product

was purified by flash chromatography (EtOAc/hexanes = 1/3) to afford compound **25** as a white foam.

TLC: 0.41 (Petroleum ether/EtOAc, 7:3)

Yield: 99%

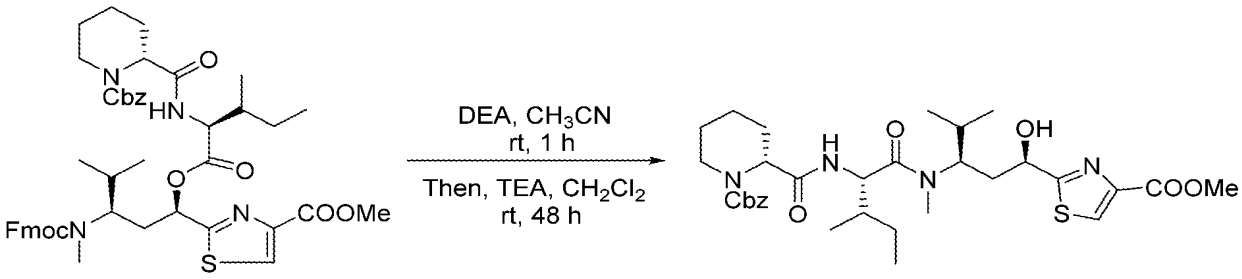
$[\alpha]_D^{25}$ = -33.2 (*C* 1, $CHCl_3$)

MS (ESI) m/z calculated for $C_{47}H_{56}N_4O_9S$ $[M+Na]^+$: 875.21, found 875.20

^1H NMR (500 MHz, $CDCl_3$): δ 7.83 – 7.72 (m, 2H), 7.66 (dd, *J* = 7.4, 1.5 Hz, 1H), 7.55 – 7.40 (m, 4H), 7.37 – 7.28 (m, 5H), 7.16 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.07 (td, *J* = 7.5, 1.5 Hz, 1H), 5.89 (t, *J* = 7.0 Hz, 1H), 4.79 (dd, *J* = 6.9, 1.1 Hz, 1H), 4.70 (d, *J* = 7.0 Hz, 2H), 4.44 (dt, *J* = 12.6, 7.0 Hz, 1H), 4.35 (t, *J* = 7.2 Hz, 1H), 4.26 (t, *J* = 6.9 Hz, 1H), 4.13 (ddd, *J* = 11.2, 7.0, 4.2 Hz, 1H), 3.96 (s, 3H), 3.16 (s, 3H), 2.81 – 2.71 (m, 2H), 2.67 – 2.45 (m, 3H), 2.02 – 1.80 (m, 4H), 1.68 – 1.55 (m, 1H), 1.55 – 1.39 (m, 3H), 1.09 (t, *J* = 8.0 Hz, 3H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.72 (dd, *J* = 24.0, 6.8 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 172.1, 171.0, 169.6, 162.4, 157.2, 156.0, 139.1, 137.1, 128.5, 128.0, 127.0, 126.8, 123.8, 122.7, 118.8, 67.4, 66.7, 63.8, 59.8, 55.6, 53.5, 52.0, 47.9, 43.3, 35.8, 35.5, 26.6, 24.5, 24.3, 20.8, 19.1, 15.0, 11.6.

Example 20: Fmoc deprotection and acyl migration: synthesis of 26



To a solution of **25** (1.0 mmol) in CH_3CN (12 mL) at 0 °C under Ar was added Et_2NH (8 mL). The solution was stirred at room temperature for 1 h. After complete deprotection of Fmoc group (TLC analysis) the mixture was evaporated and co-evaporated with CH_2Cl_2 to remove DEA. The crude crude product which was dissolved in CH_2Cl_2 (0.8 mL) and Et_3N (0.3 mL) was added and the resulting mixture was stirred at room temperature for 18 h. The crude hydroxyl derivative **26** was purified by column chromatography. The non-acylated amine was isolated and again stirred in presence of Et_3N for overnight.

TLC: 0.32 (Petroleum ether/EtOAc, 3:7)

Yield: 75%

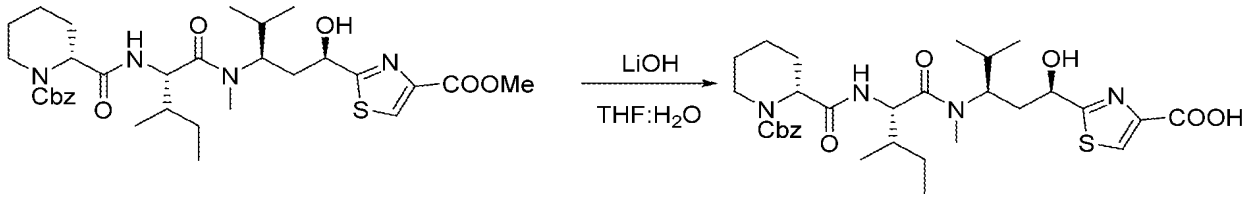
$[\alpha]_D^{25}$ = +68.2 (*C* 1, MeOH)

MS (ESI) m/z calculated for $C_{32}H_{46}N_4O_7S$ $[M+Na]^+$: 653.29, found 653.10

Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.37 – 7.29 (m, 5H), 5.54 (d, *J* = 12.4 Hz, 1H), 5.38 – 5.31 (m, 1H), 5.12 (d, *J* = 6.7 Hz, 1H), 4.83 (td, *J* = 6.9, 4.6 Hz, 2H), 4.17 (dt, *J* = 12.4, 7.1 Hz, 1H), 3.96 (s, 3H), 3.82 (ddd, *J* = 11.7, 7.0, 2.7 Hz, 1H), 3.42 (dt, *J* = 12.6, 7.1 Hz, 1H), 3.23 (d, *J* = 4.9 Hz, 1H), 2.98 (s, 3H), 2.65 – 2.55 (m, 2H), 2.54 – 2.43 (m, 1H), 2.13 (dq, *J* = 13.0, 6.9 Hz, 1H), 1.99 – 1.78 (m, 2H), 1.77 – 1.37 (m, 6H), 0.92 (d, *J* = 6.8 Hz, 3H), 0.83 (d, *J* = 6.5 Hz, 3H), 0.71 (t, *J* = 7.9 Hz, 3H), 0.59 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (125 MHz, MeOD): δ 173.9, 171.0, 170.7, 162.5, 157.2, 144.8, 137.1, 128.5, 128.0, 118.8, 66.7, 63.5, 56.7, 55.6, 54.6, 52.0, 43.3, 37.4, 36.0, 31.1, 30.8, 26.6, 24.5, 24.3, 20.8, 19.1, 14.8, 11.7.

Example 21: Hydrolysis of methyl ester: Synthesis of acid 27



26 27

LiOH (2.0 mmol) was added to a solution of **26** in a mixture of THF and H_2O (1:1, 0.5 M), and the reaction mixture was stirred at room temperature for 6 h. The mixture was concentrated under reduced pressure. The crude product was purified through silica get column chromatography.

TLC: 0.25 (CH_2Cl_2/MeOH, 9:1)

Yield: 99%

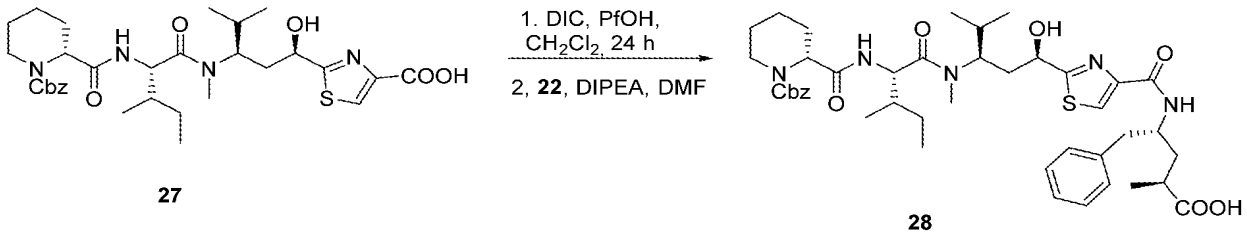
$[\alpha]_D^{25}$ = +18.5 (*C* 1, MeOH)

MS (ESI) m/z calculated for $C_{31}H_{44}N_4O_7S$ $[M+H]^+$: 617.32, found 617.15

Major rotamer: ^{1}H NMR (500 MHz, MeOD): δ 7.37 – 7.28 (m, 5H), 5.69 (d, *J* = 12.5 Hz, 1H), 5.35 – 5.22 (m, 2H), 4.94 (d, *J* = 7.0 Hz, 1H), 4.85 (t, *J* = 6.9 Hz, 1H), 4.20 (dt, *J* = 12.4, 7.0 Hz, 1H), 3.67 (ddd, *J* = 7.1, 3.8, 2.3 Hz, 1H), 3.47 (dt, *J* = 12.4, 7.0 Hz, 1H), 3.23 (d, *J* = 4.9 Hz, 1H), 2.95 (s, 3H), 2.80 (ddd, *J* = 12.4, 7.0, 2.4 Hz, 1H), 2.59 (dp, *J* = 13.7, 6.8 Hz, 1H), 2.38 (ddd, *J* = 12.3, 7.0, 3.8 Hz, 1H), 2.04 (dq, *J* = 12.7, 6.9 Hz, 1H), 1.95 – 1.78 (m, 2H), 1.78 – 1.66 (m, 1H), 1.66 – 1.55 (m, 2H), 1.52 – 1.39 (m, 2H), 1.26 (tq, *J* = 12.6, 8.0 Hz, 1H), 0.98 (d, *J* = 6.8 Hz, 3H), 0.82 (dd, *J* = 13.2, 6.8 Hz, 6H), 0.71 (t, *J* = 8.0 Hz, 3H).

Major rotamer: ^{13}C NMR (125 MHz, MeOD): δ 173.9, 173.0, 171.0, 162.3, 157.2, 150.6, 137.1, 128.5, 128.0, 122.1, 66.7, 63.5, 56.7, 55.6, 54.6, 43.3, 37.4, 36.0, 31.1, 30.8, 26.6, 24.5, 24.3, 20.8, 19.1, 14.8, 11.7.

Example 22: Coupling of Tubuphenylalanine: Synthesis of 28



27 28

Acid **27** (1.0 mmol) was added to a 0.2 M solution of pentafluorophenol (1.25 mmol) and DIC (1.0 mmol) in CH_2Cl_2 at 0 °C. The reaction mixture was warmed to rt, stirred for 24 h, and concentrated under reduced pressure. EtOAc (10 mL) was added, and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated under reduced pressure, and the crude material was used without further purification. DMF (0.335 mL, 0.25 M) was added to the crude product, followed by the hydrochloride salt of tubuphenylalanine (**22**) (2.0 mmol) and diisopropylethylamine (4.0 mmol). The reaction mixture was stirred for 24 h at rt, and DMF was removed under vacuum. The crude product was purified by column chromatography (5% CH_2Cl_2:MeOH) afforded **28** as white solid.

TLC: 0.32 (CH_2Cl_2/MeOH/TFA, 9:1:0.1)

Yield: 90%

$[\alpha]_D^{25}$ = +75.2 (*C* 1, MeOH)

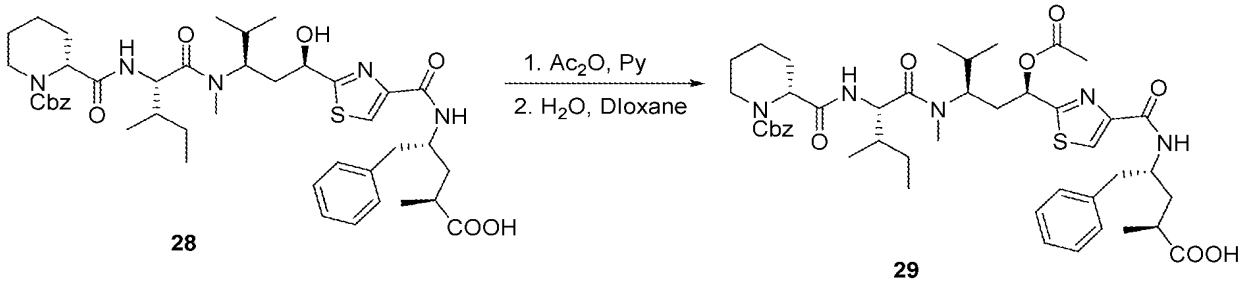
MS (ESI) m/z calculated for $C_{43}H_{59}N_5O_8S$ $[M+H]^+$: 806.10, found 806.21

Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.38 – 7.24 (m, 7H), 7.24 – 7.13 (m, 3H), 6.04 (d, *J* = 12.4 Hz, 1H), 5.14 – 5.05 (m, 3H), 4.69 (t, *J* = 6.9 Hz, 1H), 4.33 (dt, *J* = 12.5, 7.1 Hz, 1H), 4.11 (dtd, *J* = 11.5, 7.1, 1.0 Hz, 1H), 3.57 (ddd, *J* = 6.9, 4.2, 2.0 Hz, 1H), 3.42 (dt, *J* = 12.4, 7.0 Hz, 1H), 3.23 (d, *J*

= 4.9 Hz, 1H), 3.15 (ddt, *J* = 12.3, 6.9, 1.0 Hz, 1H), 2.97 – 2.84 (m, 4H), 2.79 (ddd, *J* = 12.4, 7.0, 2.0 Hz, 1H), 2.69 – 2.61 (m, 1H), 2.61 – 2.53 (m, 2H), 2.36 (ddd, *J* = 12.3, 7.0, 4.3 Hz, 1H), 2.12 (td, *J* = 11.7, 6.5 Hz, 1H), 2.01 – 1.54 (m, 7H), 1.49 (dp, *J* = 12.6, 6.8 Hz, 1H), 1.34 – 1.20 (m, 4H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.81 (dd, *J* = 22.5, 6.8 Hz, 6H), 0.71 (t, *J* = 8.0 Hz, 3H).

Major rotamer: ^{13}C NMR (125 MHz, $CDCl_3$): δ 179.2, 173.9, 171.0, 167.6, 161.5, 157.2, 150.4, 137.1, 129.3, 128.5, 128.0, 126.3, 118.6, 66.7, 63.5, 56.7, 55.6, 54.6, 53.8, 43.3, 39.3, 37.6, 37.4, 36.0, 31.1, 30.8, 26.6, 24.5, 24.3, 20.8, 19.1, 18.3, 14.8, 11.7.

Example 23: Acetylation of hydroxyl group: Synthesis of 29



A 0.1 M solution of **28** (1.0 mmol) in pyridine (10.0 mmol) was cooled to 0 °C, and acetic anhydride (10.0 mmol) was added. The reaction mixture was allowed to warm to rt over 2 h and was stirred at rt for 24 h. The reaction mixture was then cooled to 0 °C, and a 1:1 mixture of dioxane/water (4 mL) was added. The mixture was allowed to warm to rt, and was stirred for 12 h at rt. The solvent was removed under reduced pressure. Column chromatography (100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2) afforded **29** as an amorphous solid.

TLC: 0.50 (CH2Cl2/MeOH/ TFA, 9:1:0.1)

Yield: 95%

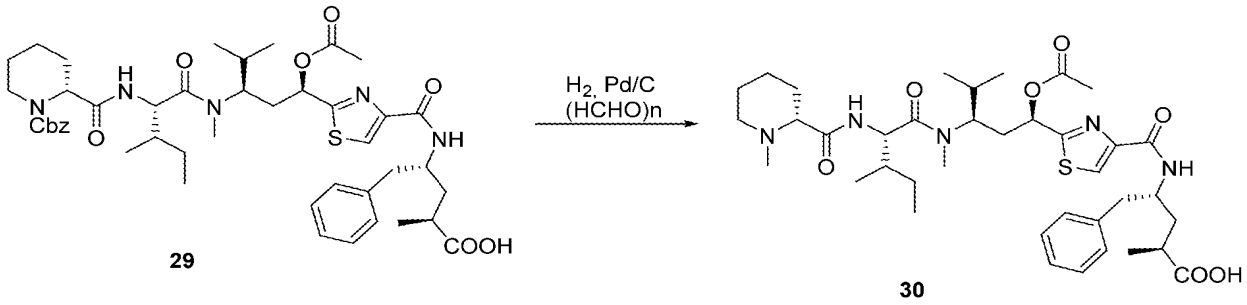
$[\alpha]_D^{25}$ = +26.5 (*C* 1, MeOH)

MS (ESI) m/z calculated for $C_{45}H_{61}N_5O_9S$ $[M+H]^+$: 848.41, found 848.35

Major rotamer: ^{1}H NMR (500 MHz, MeOD): δ δ 7.38 – 7.24 (m, 7H), 7.24 – 7.13 (m, 3H), 5.68 (t, *J* = 7.0 Hz, 1H), 5.52 (d, *J* = 12.4 Hz, 1H), 5.42 – 5.35 (m, 1H), 4.92 (t, *J* = 7.0 Hz, 1H), 4.68 (d, *J* = 6.9 Hz, 1H), 4.24 (dt, *J* = 12.6, 7.1 Hz, 1H), 4.03 (ddd, *J* = 12.6, 7.0, 3.4 Hz, 1H), 3.92 (dtd, *J* = 10.5, 7.0, 1.8 Hz, 1H), 3.54 – 3.46 (m, 1H), 3.28 (dt, *J* = 12.4, 7.0 Hz, 1H), 3.09 (ddd, *J* = 11.7, 7.1, 3.4 Hz, 1H), 2.95 (s, 3H), 2.82 (ddt, *J* = 12.4, 7.0, 1.0 Hz, 1H), 2.59 (dp, *J* = 13.6, 6.8 Hz, 1H), 2.46 (h, *J* = 6.9 Hz, 1H), 2.20 – 2.12 (m, 5H), 2.10 – 1.93 (m, 3H), 1.93 – 1.82 (m, 1H), 1.77 – 1.51 (m, 4H), 1.39 (dp, *J* = 13.0, 7.0 Hz, 1H), 1.26 (d, *J* = 6.8 Hz, 3H), 1.17 (tq, *J* = 13.0, 8.2 Hz, 1H), 0.93 (d, *J* = 6.8 Hz, 3H), 0.77 – 0.67 (m, 9H).

Major rotamer: ^{13}C NMR (125 MHz, $CDCl_3$): δ 179.2, 173.9, 171.0, 170.5, 164.6, 161.5, 157.2, 150.4, 137.1, 129.3, 128.5, 128.0, 126.3, 118.6, 66.7, 62.5, 56.7, 55.6, 54.2, 53.8, 43.3, 39.3, 37.6, 37.2, 36.0, 35.8, 31.1, 30.8, 26.6, 24.5, 24.3, 21.3, 20.8, 19.1, 18.3, 14.8, 11.7.

Example 24: Cbz deprotection and N-methylation: Synthesis of 30



The compound **29** (1.0 mmol) was dissolved in a mixture of MeOH (20 ml). Paraformaldehyde (300 mg, 10 mmol) and 20% Pd/C (106 mg, 0.1 mmol Pd) were added. The reaction mixture was stirred under hydrogen atmosphere for 16 h and afterwards filtered through Cellite. The solvent was then removed under reduced pressure. The product was purified through column chromatography.

TLC: 0.51 (CH_2Cl_2/MeOH/TFA; 9:1:0.1).

Yield: 86%

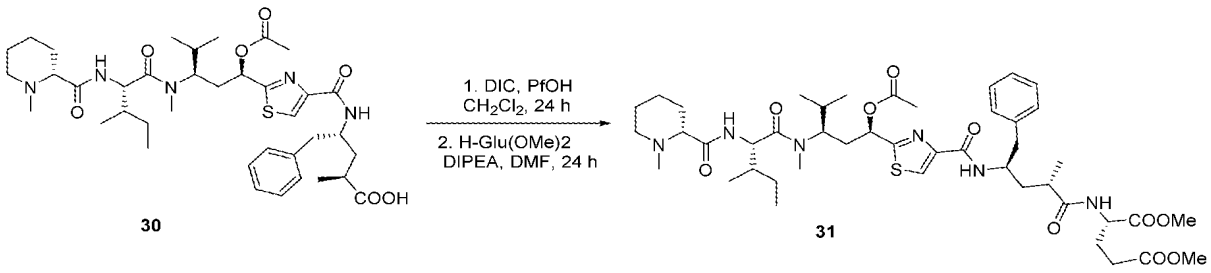
$[\alpha]_D^{25}$ = + 19.2 (c=1.0, MeOH).

MS (ESI) m/z calculated for $C_{38}H_{57}N_5O_7S$ $[M+H]^+$: 728.40, found 728.35.

^1H NMR (500 MHz, MeOD): δ 8.08 (s, 1H), 7.19–7.25 (m, 4H), 7.13–7.18 (m, 1H), 5.71 (dd, 1H, *J* = 2.5, 11.0 Hz), 4.73 (d, 1H, *J* = 8.0 Hz), 4.30–4.50 (m, 2H), 3.10 (s, 3H), 3.05 (d, 1H, *J* = 11.5 Hz),), 2.92 (d, 2H, *J* = 6.5 Hz), 2.85 (d, 1H, *J* = 10.5 Hz, 2.51 (br s, 1H), 2.23–2.41 (m, 3H), 2.31 (s, 3H), 2.15 (s, 3H), 1.96–2.05 (m, 1H), 1.75–1.92 (m, 4H), 1.56–1.74 (m, 5H), 1.37–1.41 (m, 1H), 1.09–1.23 (m, 1H), 1.16 (d, 3H, *J* = 7.0 Hz), 1.03 (d, 3H, *J* = 6.5 Hz), 0.98 (d, 3H, *J* = 6.5 Hz), 0.92 (t, 3H, J=7.3 Hz), 0.81 (d, 3H, *J* = 6.5 Hz).

^{13}C NMR (125 MHz, MeOD): δ = 11.3, 16.4, 19.1, 20.4, 20.6, 20.9, 23.7, 25.5, 25.5, 30.9, 31.0, 31.1, 35.6, 37.6, 39.5, 39.6, 42.0, 44.2, 51.2, 55.2, 56.4, 69.7, 71.2, 125.1, 127.4, 129.3, 130.6, 139.8, 151.1, 162.7, 171.6, 171.8, 173.6, 175.0, 182.5 ppm.

Example 25 : Synthesis of tubulysin conjugate.



30 31

Acid **30** (1.0 mmol) was added to a 0.2 M solution of pentafluorophenol (1.25 mmol) and DIC (1.0 mmol) in a CH_2Cl_2 at 0 °C. The reaction mixture was warmed to rt, stirred for 24 h, and concentrated under reduced pressure and dissolved in DMF (0.5 M). In another reaction vessel hydrochloride salt of H-Glu-$(OMe)_2$ (3.0 mmol) and diisopropylethylamine (5.0 mmol) were dissolved in DMF (0.335 mL, 0.25 M) and this solution is added to the above prepared pentaflurophenyl ester. The reaction mixture was stirred for 24 h at rt, and DMF was removed under vacuum. The crude product was purified by RP-

semi HPLC (20-80% CH_3CN in H_2O for 30 min at 214 nm) afforded **31** as white off solid.

HPLC analysis: t_R = 3.44 min (Chromolith, gradient: 20-70% B/A over 5 min)

HPLC purification: Nucleosil C18, gradient: 20-21% B/A over 12 min, 25 °C

Yield: 75%

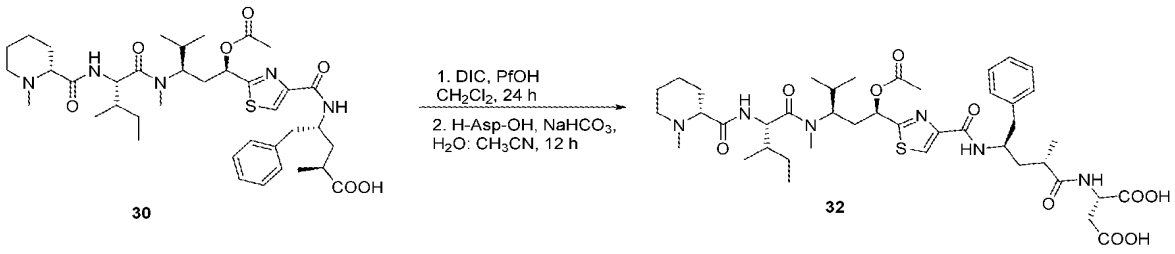
$[\alpha]_D^{25}$ = +75.2 (*C* 1, MeOH)

MS (ESI) m/z calculated for $C_{45}H_{68}N_6O_{10}S$ $[M+Na]^+$: 907.46, found 907.40

Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.41 – 7.16 (m, 7H), 7.12 – 7.00 (m, 3H), 6.98 (d, *J* = 18.1 Hz, 1H), 6.04 (d, *J* = 12.4 Hz, 1H), 5.55 (br, s, 1H), 5.25 – 5.20 (m, 3H), 4.25 (t, *J* = 6.9 Hz, 1H), 4.23 – 4.10 (m, 3H), 3.65 (s, 3H), 3.61 (s, 3H), 3.57 – 3.55 (m, 1H), 3.42 (dt, *J* = 12.4, 7.0 Hz, 1H), 3.23 (d, *J* = 4.9 Hz, 1H), 3.15 (ddt, *J* = 12.3, 6.9, 1.0 Hz, 1H), 3.10 – 3.00 (m, 2H), 2.97 – 2.84 (m, 4H), 2.79 – 2.72 (m, 3H), 2.69 – 2.61 (m, 1H), 2.61 – 2.53 (m, 2H), 2.36 (ddd, *J* = 12.3, 7.0, 4.3 Hz, 1H), 2.12 (td, *J* = 11.7, 6.5 Hz, 1H), 2.01 – 1.54 (m, 7H), 1.49 (d, *J* = 12.6, 6.8 Hz, 1H), 1.34 – 1.20 (m, 4H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.81 (dd, *J* = 22.5, 6.8 Hz, 6H), 0.71 (t, *J* = 8.0 Hz, 3H).

Major rotamer: ^{13}C NMR (125 MHz, $CDCl_3$): δ 180.2, 179.4, 178.2, 178.0, 173.0, 171.5, 166.3, 161.9, 157.0, 150.5, 137.4, 129.0, 128.2, 128.0, 126.1, 118.1, 66.1, 63.7, 56.7, 55.5, 54.5, 53.0, 48.8, 45.5, 43.0, 39.7, 37.9, 36.5, 36.1, 31.1, 30.8, 26.7, 24.0, 24.1, 20.7, 19.1, 18.3, 14.8, 12.5, 11.9, 11.2.

Example 26 : Synthesis of tubulysin conjugate



Acid **30** (1.0 mmol) was added to a 0.2 M solution of pentafluorophenol (1.25 mmol) and DIC (1.0 mmol) in a CH_2Cl_2 at 0 °C. The reaction mixture was warmed to rt, stirred for 24 h, and concentrated under reduced pressure and dissolved in acetonitrile (0.5 M). In another reaction vessel (L) H--Asp-OH, (2.0 mmol) and sodium bicarbonate (4.0 mmole) were dissolved in 2 ml. of water and adjust the pH about 8. Then a solution of pentafluorophenyl ester prepared above (1.0 mmole) 3 mL of acetonitrile was added at room temperature. The reaction mixture was stirred overnight. The solvent were removed under vacuum. The crude product was purified by RP-semi HPLC (20-60% CH_3CN in H_2O for 30 min at 214 nm) afforded **32** as white off solid.

HPLC analysis: t_R = 2.82 min (Chromolith, gradient: 20-60% B/A over 5 min)

HPLC purification: Nucleosil C18, gradient: 20-60% B/A over 30 min

Yield: 68 %

$[\alpha]_D^{25}$ = + 125.6 (*C* 1, MeOH)

MS (ESI) m/z calculated for $C_{42}H_{62}N_6O_{10}S$ $[M+H]^+$: 842.42, found 842.40

Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.38 – 7.19 (m, 7H), 7.10 – 6.89 (m, 3H), 6.80 (d, *J* = 18.1 Hz, 1H), 6.55 (d, *J* = 12.4 Hz, 1H), 5.89 (br, s, 1H), 5.11 – 5.00 (m, 3H), 4.25 (t, *J* = 6.9 Hz, 1H), 4.32 – 4.13 (m, 3H), 3.55 – 3.38 (m, 1H), 3.40 (dt, *J* = 12.4, 7.0 Hz, 1H), 3.23 (d, *J* = 4.9 Hz, 1H), 3.15 (ddt, *J* = 12.3, 6.9, 1.0 Hz, 1H), 3.10 – 2.90 (m, 2H), 2.80 – 2.71 (m, 2H), 2.66 – 2.62 (m, 3H), 2.55 – 2.45 (m, 1H), 2.40 – 2.33 (m, 2H), 2.30 (ddd, *J* = 12.3, 7.0, 4.3 Hz, 1H), 2.10 (td, *J* = 11.7, 6.5 Hz, 1H), 1.96 – 1.64 (m, 7H), 1.52 (d,

J = 12.6, 6.8 Hz, 1H), 1.41 – 1.30 (m, 4H), 0.94 (d, J = 6.8 Hz, 3H), 0.81 (dd, J = 22.5, 6.8 Hz, 6H), 0.71 (t, J = 8.0 Hz, 3H).

Major rotamer: ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.6, 180.1, 177.1, 172.0, 171.6, 165.3, 160.9, 155.2, 150.8, 138.2, 128.0, 127.2, 127.4, 126.2, 118.4, 65.8, 59.7, 55.7, 53.4, 53.7, 48.6, 45.0, 43.7, 38.1, 37.7, 37.5, 35.7, 31.0, 30.2, 26.7, 25.8, 24.1, 21.5, 19.8, 18.0, 13.6, 12.1, 11.0.

It was found that compound **30** (100 nM test concentration) is highly toxic to HeLa cells after 18hrs treatment, thus demonstrating its suitability as cytotoxic agent e.g. in targeted drug delivery.

Example 27: Further tubulysin derivatives

A person skilled in the art will understand and appreciate that the present invention relies on two parts. One part is the Passerini MCR- based approach to construct a tetrapeptide adduct in a good yield using only three synthetic steps.

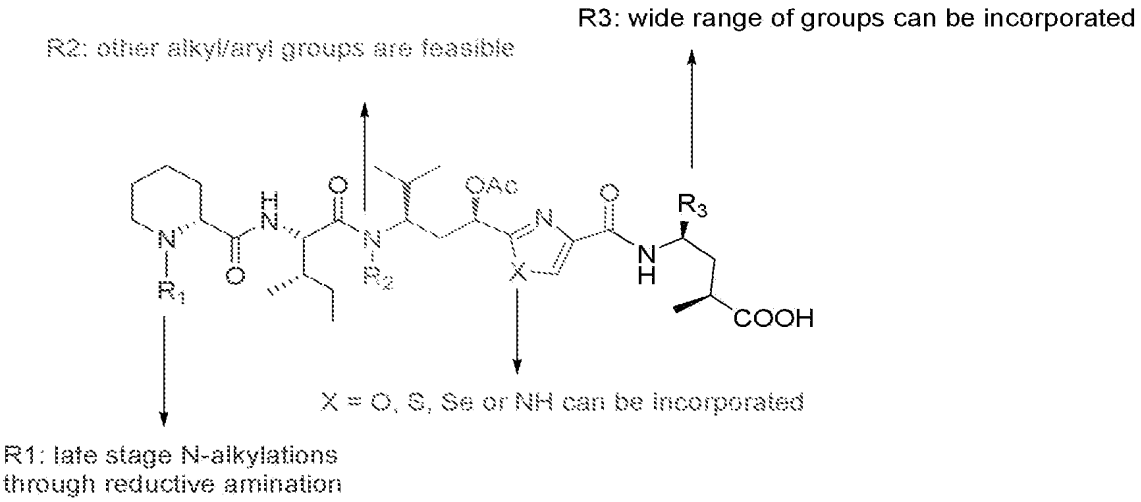
Secondly, as is exemplified by the synthesis of Tubuphenylalanine (Tup; general formula G), the invention uses simple reagents and less steps to obtain Tup is a highly diastereoselective manner. Another major advantage of this method is that, any other unnatural γ-amino acids are easily obtainable by the simple Grignard reaction. This diversity has never been studied so far.

The tubulysin "derivative" is therefore considered in the broad context and allows for:

- Variation on the *N*-alkylated Tuv substituents (Not only limited to *N*-Me),
- Diversity possible from the X-substituents from the isocyanide

- Higher alkyl chains (not only *N*-Me) can be incorporated on the N-terminal Picolinic acid
- Wide range of Tup derivatives are also constructed

See the following scheme:

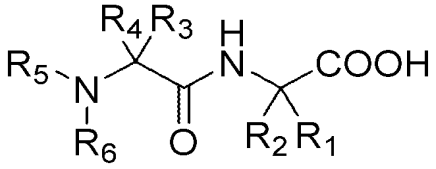


All these variations can be easily adopted through the standard protocol as herein disclosed, thus allowing to obtain a wide range of tubulysin derivatives.

Claims

1. A method for preparing a tubulysin derivative, comprising reacting compounds A, B and C in a 3-component Passerini reaction,

wherein compound A is a carboxylic acid according to the general formula A



wherein

R_1 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; or a substituted or unsubstituted benzyl,

R_2 represents H, a substituted or unsubstituted alkyl, or a substituted or unsubstituted cycloalkyl;

R_3 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; or a substituted or unsubstituted benzyl;

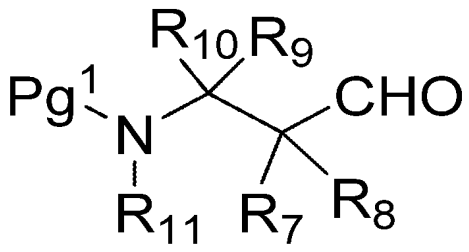
R_4 represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, a substituted or unsubstituted benzyl;

R_5 represents a substituted or unsubstituted alkyl or a substituted or unsubstituted cycloalkyl; preferably a substituted or unsubstituted cycloalkyl;

or wherein R_4 and R_5 are connected to form a 4- to 7-membered ring;

R_6 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; a substituted or unsubstituted benzyl, or C=OOR', where R' is an optionally substituted alkyl, cycloalkyl, benzyl or aroyl moiety;

wherein compound B is an aldehyde according to the general formula B

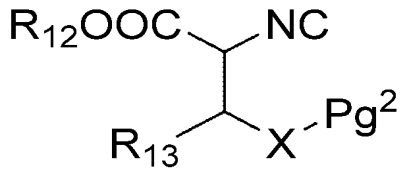


wherein R_7, R_8, R_9, R_{10} and R_{11} each independently represent H, F, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl or a substituted or unsubstituted benzyl;

Pg^1 is an amine protecting group, preferably a carbamate, a substituted or an unsubstituted benzyl, or a substituted or an unsubstituted sulfonamide;

and

wherein compound C is an isocyanide according to the general formula C



wherein

R_{12} represents a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

R_{13} represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

X represents O, S, Se, or -NH-, preferably S; and

Pg^2 represents an X- protecting group, preferably selected from trityl, tert-butyl, adamantyl and substituted benzyl, more preferably trityl or tert-butyl.

2. Method according to claim 1, wherein

R_1 represents isopropyl, tert-butyl, iso-butyl, sec-butyl, cyclopropylmethyl or cyclobutylmethyl;

R_2 is H;

R_3 is $-(CH_2)_n-CH_3$, wherein n is 3 to 5;

R_4 is CH_2-CH_2- or $-CH_2-CH_2-CH_2-$ connected to R_5;

R_5 is a substituted or unsubstituted cycloalkyl; and/or

R_6 is selected from the group consisting of benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate and ethyl carbamate.

3. Method according to claim 1 or 2, wherein

R_7 is H;

R_8 is H;

R_9 is selected from the group consisting of isopropyl, cyclopropyl, cyclobutyl, isobutyl, sec-butyl, tert-butyl and cyclopropylmethyl;

R_{10} is H; and/or

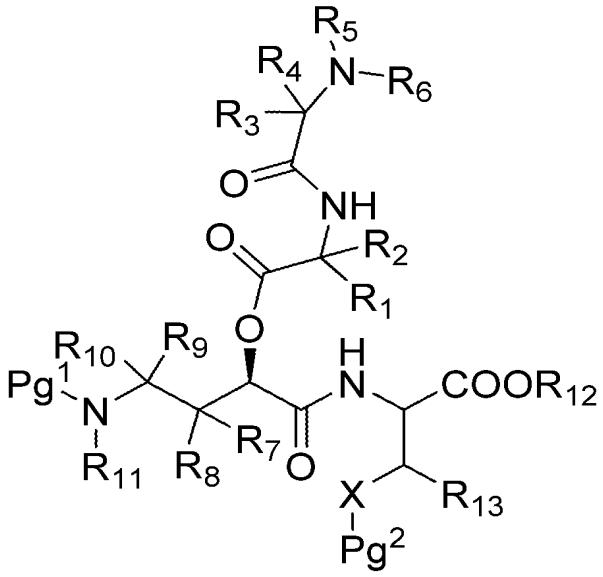
R_{11} is selected from the group consisting of methyl, ethyl, propyl, butyl, isopropyl, cyclopropyl and cyclopropylmethyl.

4. Method according to any one of the preceding claims, wherein Pg^2 is selected from the group consisting of benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate, ethyl carbamate, benzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl.

5. Method according to any one of the preceding claims, wherein R_{12} is methyl, ethyl or tert-butyl; and/or wherein R_{13} is H or methyl.

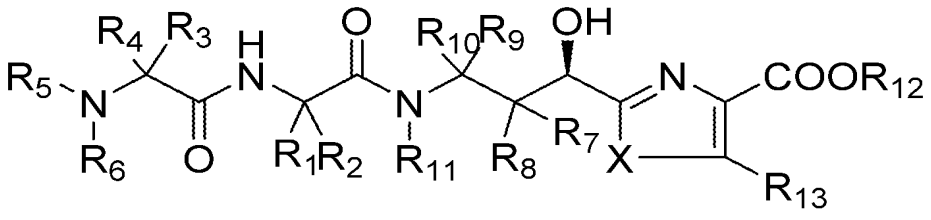
6. Method according to any one of the preceding claims, comprising reacting compounds A, B and C in a non-coordinating solvent or solvent mixture, preferably selected from the group consisting of CH_2Cl_2, $CHCl_3$, CCl_4, benzene, THF, CH_3CN, 1,4-dioxane, 1,2-dichloroethane and a mixture of CH_2Cl_2:THF (1:1 v/v).

7. Method according to any one of the preceding claims, further comprising isolating the 3-component Passerini reaction product of Formula D



wherein each of the substituents is as defined in claims 1-5.

8. Method according to claim 7, further comprising subjecting the Passerini reaction product of formula D to (a) an acyl migration reaction and (b) a cyclodehydration reaction of the Cys-amide to obtain a compound of the general formula E



9. Method according to claim 8, wherein said acyl migration reaction is performed in a two-step process involving exposure to diethylamine (DEA) followed by exposure to trimethylamine (TEA).

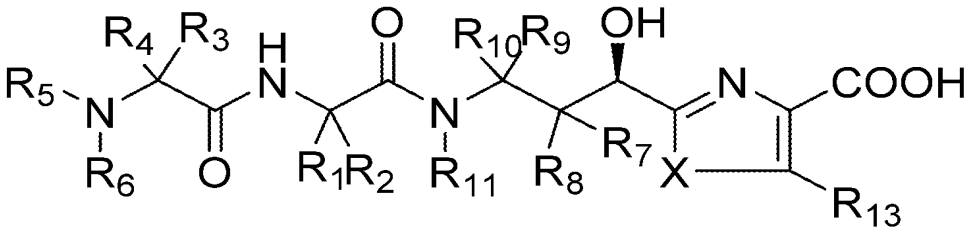
10. Method according to claim 8 or 9, wherein said cyclodehydration reaction is performed in a two-step process involving incubation in the

presence of $TiCl_4$, followed by oxidation in the presence of MnO_2, preferably activated MnO_2 having a pore size of ≤ 5 microns.

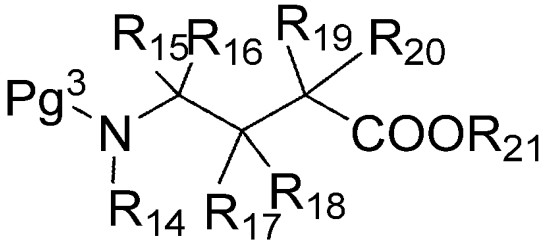
11. Method according to any one of claims 8-10, wherein acyl migration precedes cyclodehydration.

12. Method according to any one of claims 8-10, wherein cyclodehydration precedes acyl migration.

13. Method according to any one of claims 8 to 12, further comprising hydrolyzing the ester of the general formula E to obtain the carboxylic acid compound of the general formula F



14. Method according to claim 13, further comprising reacting the carboxylic acid compound of the formula F with a compound of the general formula G



wherein

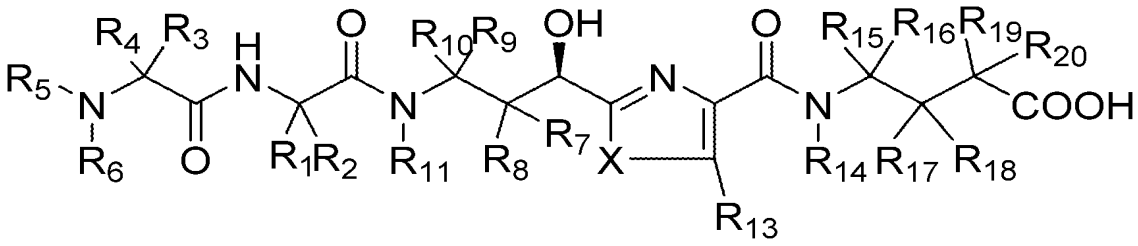
R_{14}, R_{15}, R_{16}, R_{20} and R_{21} each independently represent H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

R_{17} and R_{18} each independently represent H, F, a substituted or unsubstituted alkyl, or a substituted or unsubstituted cycloalkyl;

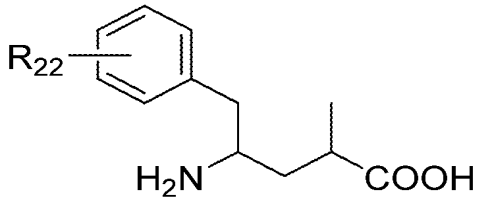
R_{19} represents H, F, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl; and

Pg^3 is an amine protecting group, preferably a carbamate, a substituted or unsubstituted benzyl, preferably selected from the group consisting of tertbutyl sulfine, benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate, ethyl carbamate, benzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl

followed by removal of protecting moiety R_{21},
to obtain a compound of the general formula H



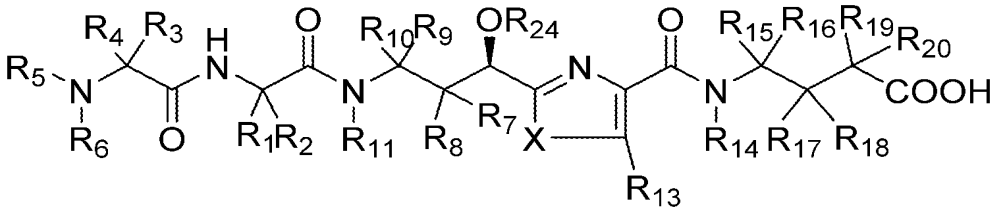
15. Method according to claim 14, wherein the compound of the general formula G is tubuphenylalanine of the formula.



or a salt thereof,

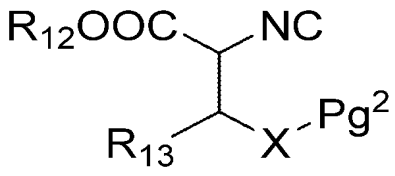
wherein R_{22} is H, OH, F or NO_2, preferably H, OH or F.

16. Method according to claim 14 or 15, further comprising the steps of acylation of the hydroxyl group of the general formula H to obtain a compound of the general Formula I



wherein R_{24} represent acetyl, acyl (substituted) alkyl, acyl cycloalkyl, or acyl benzyl, preferably acetyl or acyl derivative of methyl, ethyl, tert-butyl or benzyl.

17. An isocyanide compound according to the general formula C



wherein

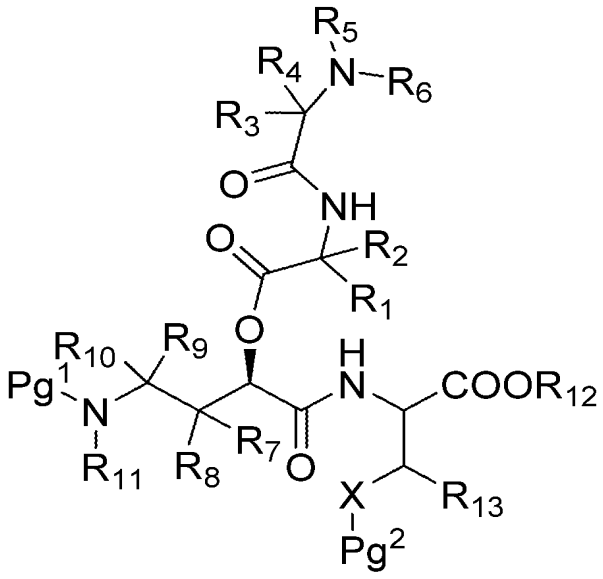
R_{12} represents a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

R_{13} represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

X represents O, S, Se, or -NH-, preferably S; and

Pg^2 represents a -X protecting group, preferably selected from the group consisting of trityl, tert-butyl, adamantly, and a substituted benzyl, more preferably trityl or tert-butyl.

18. The 3-component Passerini reaction product obtainable by the method of claim 7, having the general formula



wherein each of the substituents is as defined in claims 1-5.

19. The use of an isocyanide compound according to claim 17 in the manufacture of a tubulysin derivative.

20. The use of the Passerini reaction product according to claim 18 in the manufacture of a tubulysin derivative.

21. The use according to claim 19 or 20, in the manufacture of a tubulysin-prodrug or a tubulysin ADC.

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2019/050481

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K5/02 A61K47/54 A61K47/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THIMMALAPURA M. VISHWANATHA ET AL: "Cysteine Isocyanide in Multicomponent Reaction: Synthesis of Peptido-Mimetic 1,3-Azoles", JOURNAL OF ORGANIC CHEMISTRY, vol. 82, no. 18, 25 August 2017 (2017-08-25), pages 9585-9594, XP055544660, ISSN: 0022-3263, DOI: 10.1021/acs.joc.7b01615 compound 4 ----- -/--	17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 November 2019	13/11/2019

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2019/050481

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XUECHEN LI ET AL: "On the Two-Component Microwave-Mediated Reaction of Isonitriles with Carboxylic Acids: Regarding Alleged Formimidate Carboxylate Mixed Anhydrides", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 130, no. 40, 8 October 2008 (2008-10-08), pages 13222-13224, XP055544845, ISSN: 0002-7863, DOI: 10.1021/ja8047078 compound 27	17
X	TAKUYA FUKUDA ET AL: "Anti-barnacle Activity of Isocyanides Derived from Amino Acids", CHEMISTRY & BIODIVERSITY, vol. 13, no. 11, 1 November 2016 (2016-11-01), pages 1502-1510, XP055544849, CH ISSN: 1612-1872, DOI: 10.1002/cbdv.201600063 compound 6	17
A	EP 2 409 983 A1 (LEIBNIZ INST FUER PFLANZENBIOCHEMIE IPB [DE]) 25 January 2012 (2012-01-25) paragraph [0007] - paragraph [0009] paragraph [0014] - paragraph [0015] figures 1, 2	1-21

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2019/050481

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2409983 A1	25-01-2012	AU 2011281939 A1	21-02-2013
		CA 2803948 A1	26-01-2012
		EP 2409983 A1	25-01-2012
		EP 2596008 A1	29-05-2013
		ES 2534068 T3	17-04-2015
		JP 5907513 B2	26-04-2016
		JP 2013536171 A	19-09-2013
		US 2013217638 A1	22-08-2013
		WO 2012010287 A1	26-01-2012